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Palau'amine and Related Oroidin Alkaloids Dibromophakellin and Dibromophakellstatin Inhibit the Human 20S Proteasome

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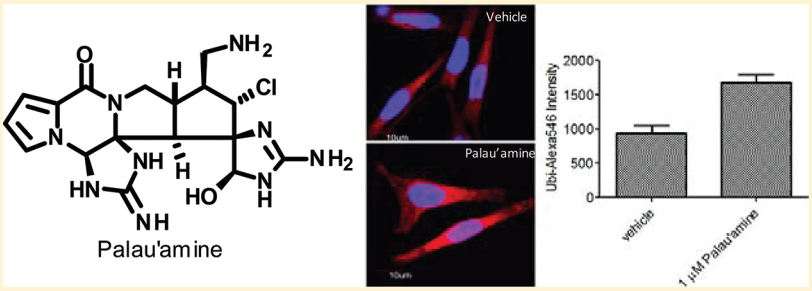
Supporting Information



ABSTRACT: We report herein that the oroidin-derived alkaloids palau'amine (**1**), dibromophakellin (**2**), and dibromophakellstatin (**3**) inhibit the proteolytic activity of the human 20S proteasome as well as the ^{i}20S immunoproteasome catalytic core. Palau'amine is found to prevent the degradation of ubiquitinylated proteins, including IκBα, in cell culture, which may be indicative of the potential mechanism by which these agents exhibit their exciting cytotoxic and immunosuppressive properties.

The oroidin family of alkaloids is a highly diverse and complex class of biologically active secondary marine sponge metabolites containing characteristic pyrrole-2-carboxamide and 2-aminoimidazoline (or derivatives thereof) moieties. Members of this group include the highly publicized palau'amine as well as the structurally related phakellins and phakellstatins. The polycyclic dimeric pyrrole-imidazole alkaloid palau'amine (**1**) was first isolated by Scheuer and co-workers in 1993[1] and structurally reassigned in 2007.[2] The unique structure and its exciting immunosuppressive and cytotoxic properties[1] have motivated many synthetic groups to embark on its total synthesis.[3] The architecturally daunting natural product finally succumbed to total synthesis by Baran and co-workers in 2010,[4] which allowed for more detailed biological evaluation.

The structurally related natural products (−)-dibromophakellin (**2**) and its enantiomer, (+)-dibromophakellin, were isolated in 1969 by Sharma[5] and in 1985 by Ahond and Poupat,[6] respectively. The total synthesis of (±)-dibromophakellin has been completed,[7−9] as well as the enantioselective synthesis of (+)-dibromophakellin.[10,11] In addition to the phakellins, (−)-dibromophakellstatin (**3**) also resembles, in part, the palau'amine core structure. (−)-Dibromophakellstatin (**3**) was first isolated in 1997 by Pettit,[12] and its total synthesis has been completed by Lindel's group.[13] The total synthesis of (±)-dibromophakellstatin also has been completed,[7,14,15] along with the asymmetric synthesis of (+)-dibromophakellstatin.[10,16] Both the phakellins and phakellstatins have been reported to possess potent cell growth inhibitory activity against a variety of cell lines,[15] via unknown mechanisms,[17] although it should be mentioned that **2** was reported to display agonist activity against the alpha(2B) adrenoceptor.[18] Synthetic efforts by Feldman's group have provided sufficient quantities of material of these natural products for further biological evaluation in search of the mechanism of action.[7,19]

Herein we report that these oroidin-derived alkaloids were found to modulate the proteolytic activity of the human proteasome, as well as the immunoproteasome. In addition, we show that palau'amine is capable of preventing the proteolytic degradation of proteins, including the inhibitory-κB protein in cell culture, which may be the underlining mechanism or at least be in part responsible for the exciting immunosuppressive and cytotoxic properties elicited by these agents.

In order to maintain biological homeostasis and regulation of intercellular processes, proteins undergo constant proteolytic degradation by the 26S proteasome. This process of protein degradation is a highly controlled and multistep process, which typically requires the ubiquitinylation of a protein target for recognition and subsequent degradation by the 26S proteasome.[20] The 26S proteasome is a multifunctional, 2.5 MDa

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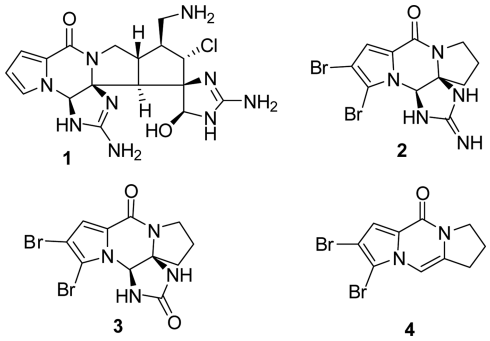
protein complex in which several enzymatic functions work together to degrade proteins.[21,22] The 26S proteasome consists of the 20S catalytic core and the 19S regulatory particles. The 20S core is responsible for protein degradation. The 19S particle is responsible for recognition, unfolding, and translocation of the substrates into the 20S catalytic core.[23] The 20S core is composed of two heptameric inner rings of β-subunits and two heptameric outer rings of α-subunits.[22] The chymotryptic-like (CT-L), tryptic-like (T-L), and caspase-like (Casp-L) catalytic sites reside in the β-subunits, and these catalytic sites are responsible for the proteolytic activity of the proteasome. While the majority of cell types express the constitutive form of the proteasome, cells of the immune system (monocytes and lymphocytes) express the immunoproteasome,[24] which incorporates the catalytic subunits LMP7, MECL1, and LMP2, instead of β5, β2, and β1, in its ^{i}20S catalytic core.[24] Nonimmune cells can in some cases also express the immunoproteasome following exposure to inflammatory cytokines, such as INF-γ or TNF-α.[25]



Disruption of proteasome-mediated protein degradation is a viable means to treat immune diseases and inflammatory disorders[26] and has been validated in the clinic in the treatment of certain cancers,[27] including multiple myeloma[28] and mantle cell lymphoma.[29] With the notable exception of the natural product salinosporamide A, nearly all clinically relevant proteasome inhibitors are peptide-based scaffolds.[27,30,31] Thus, given their clinical significance, the identification of new classes of proteasome inhibitors is highly desirable.

RESULTS AND DISCUSSION

Palau'amine (1), Dibromophakellin (2), and (−)-Dibromophakellstatin (3) Inhibit the Proteolytic Activity of the Human Proteasome. The ability of the oroidin alkaloids to inhibit the 20S proteasome was determined in vitro using purified human 20S core enzyme and the following fluorogenic peptides as substrates: Suc-LLVY-AMC (substrate for CT-L activity), Boc-LRR-AMC (substrate for T-L activity), and Z-LLE-AMC (substrate for Casp-L activity).[32] The rates of hydrolysis were monitored by fluorescence increase at 37 °C over 30 min, and the linear portion of the curve was used to calculate the IC_{50} values. Each IC_{50} is the average of three or more independent experiments (Supporting Information Tables S1−S3). These experiments demonstrate that racemic palau'amine inhibited the CT-L activities (IC_{50} 5.5 μM, Table 1) and Casp-L activities (IC_{50} 3.0 μM, Table 1) of the 20S catalytic core. Palau'amine did not affect T-L activity of the proteasome. The natural (−)-enantiomer of palau'amine exhibited an IC_{50} of approximately half of the racemate (IC_{50} 2.5 μM for CT-L activity and 1.6 μM for Casp-L activity, Table 1), indicating that the inhibitory activity against the 20S proteasome is primarily found in the natural stereoisomer. The related racemic dibromophakellin (**2**) and dibromophakellstatin (**3**) also inhibit the CT-L and Casp-L activities at low micromolar IC_{50} values (Supporting Information Tables S1−S3). Dibromophakellin (**2**) reduced the proteolytic activity at an IC_{50} of 25 μM. Dibromophakellstatin (**3**) exhibited an IC_{50} of 12 μM. Of particular significance is that the synthetic analogue **4**,[9] which lacks the urea or guanidine moiety, was devoid of all activity. This indicates the requirement of this fused ring system for the inhibition of the proteasome. Considering the reported immunosuppressive properties of palau'amine, we evaluated the chymotryptic-like activity of the catalytic domain of the human immunoproteasome (^{i}20S CT-L) and found that (±)-palau'amine (**1**) effectively reduced its proteolytic activity at a similar potency with an IC_{50} of 4.0 μM for the racemic compound (Table 1). Consistent with the data observed for the 20S proteasome, the natural (−)-enantiomer was also found to be approximately twice as potent as the racemate toward the immunoproteasome (IC_{50} 2.3 μM, Table S4).

Table 1. Inhibition of the Chymotryptic-like (CT-L), Caspase-like (Casp-L), and Tryptic-like (T-L) Activity of Purified Human 20S Proteasome and CT-L Activity of Human ^{i}20S Immunoproteasome by Oroidin-Derived Alkaloids: Racemic Palau'amine ((±)-1), (−)-Palau'amine ((−)-1), (±)-Dibromophakellin (2), and (±)-Dibromophakellstatin (3) and Synthetic Precursor 4

compound	CT-L (μM)	Casp-L (μM)	T-L (μM)	^{i}20S CT-L (μM)
(±)-1	5.5 (±1.5)	3.0 (±0.4)	>100	4.0 (±0.4)
(−)-1	2.5 (±0.7)	1.6 (±0.8)	>100	2.3 (±0.01)
(±)-2	25.3 (±4.4)	27.1 (±6.4)	>100	18.7
(±)-3	11.9 (±0.7)	16.1 (±1.2)	>100	6.2 (±1.6)
4	>100	>100	>100	>100

Inhibition of the Proteasome Is Elicited by Palau'amine and Not Its Decomposition Products or Coagulates. Palau'amine (**1**) has been reported to be unstable at a pH of greater than 7. Considering this, we investigated the activity of the degradation products produced by palau'amine after incubation in reaction buffer at pH 7.5 and 8.0, over time. When palau'amine was incubated in assay buffer before the addition of enzyme, the inhibitory activity of palau'amine toward proteasome inhibition decreases rapidly in a time-dependent manner at pH >7.5 (Figure S1). These experiments indicate that the inhibitory activities toward the proteasome are due to the natural product itself and not its degradation products.

It is common to identify false positive inhibitors in *in vitro* assays. When amphipathic molecules are dispersed in an aqueous environment, the molecules can form colloidal aggregates. These aggregates can modulate enzyme activities by absorption onto the surface of the enzyme or by sequestering the enzyme within the colloidal aggregate. In order to eliminate the possibility of false positives due to aggregation, the critical micelle concentration of palau'amine was determined experimentally using a UV-absorption spectroscopic method based on the tautomerism of benzoylacetone.[33] (±)-Palau'amine was evaluated in this aggregation assay from 1 to 10 μM (in the range of its IC_{50} for human 20S proteasome) and was found not to form aggregates. Additional support against aggregate formation was found in centrifugation

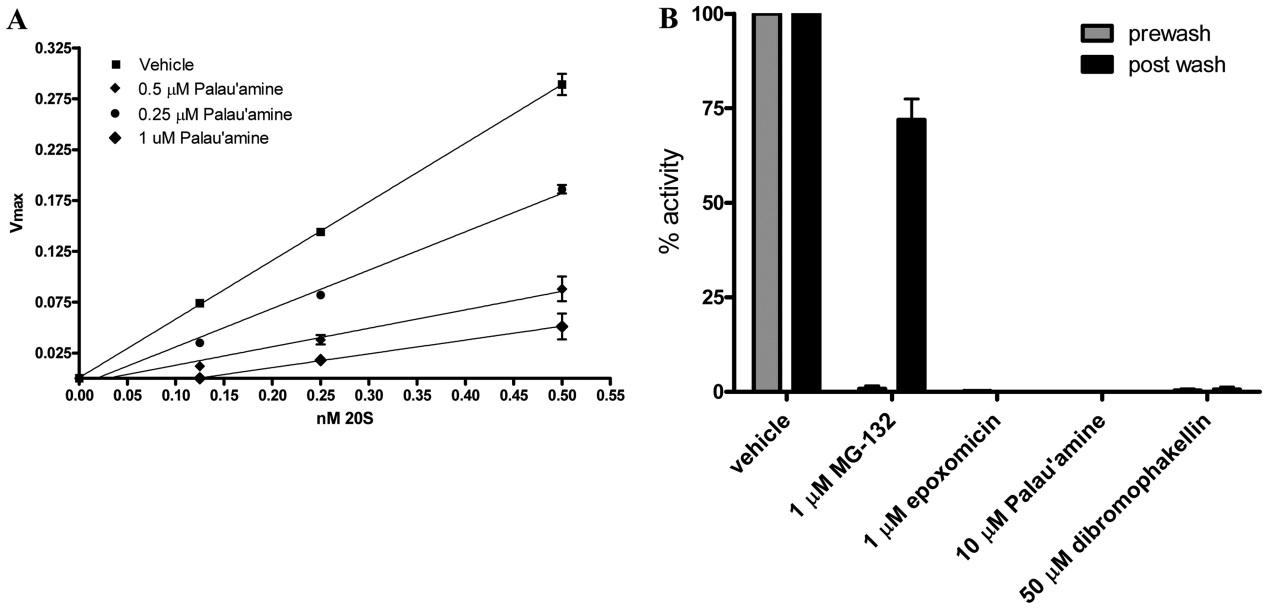


Figure 1. (A) Kinetics for inhibition of CT-L activity using varying concentrations of palau'amine (**1**). (B) Percent chymotryptic-like (CT-L) activity of 20S proteasome pre- and post-washing with 500× washing buffer after exposure to excess MG-132, epoxomicin, and palau'amine (**1**).

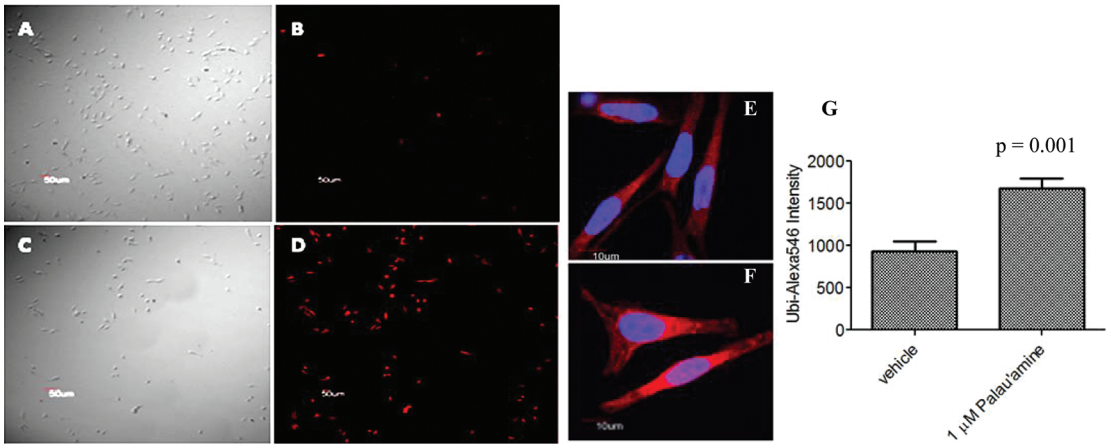


Figure 2. Ubiquitinated protein accumulation in HeLa cells. (A and B) Treatment with vehicle (0.1% DMSO). (C and D) Treatment with (±)-palau'amine (1 μM in 0.1% DMSO vehicle). Panels A and C are differential interference contrast images of the cells. The cells were treated for 2 h with either vehicle or palau'amine, fixed, and stained for ubiquitin (red) and DNA (blue). The cells were visualized by confocal microscopy with a 10× objective. (E and F) Higher magnification images (60× objective with 2× zoom) of panels B and D, respectively. Scale bars: 20 μm. (G) Quantification of fluorescent intensity of the ubiquitin−Alexa 546 conjugate.

experiments, where the IC_{50} value of palau'amine was compared pre- and post-centrifugation (at 15000*g* for 30 min) in the reaction buffer solution. During this centrifugation, any aggregates would be cleared from the supernatant and would result in an altered inhibitory profile. The supernatant was used to measure the IC_{50} of the remaining palau'amine against the CT-L activity. This resulted in no significant change in the IC_{50} value from the precentrifugated sample, thus eliminating the possibility of aggregation (Figure S2). Considering the superior potency of palau'amine over the related oroidin alkaloids dibromophakellin (**2**) and dibromophakellstatin (**3**), our further studies were focused on palau'amine.

Palau'amine Binds Irreversibly to the 20S Proteasome. Binding of palau'amine to the 20S proteasome is consistent with an irreversible-type mechanism, as indicated by both kinetic analysis (Figure 1A) and washing experiments (Figure 1B). In Figure 1A, we evaluated palau'amine at three different concentrations, with varying concentrations of purified proteasome and measured the rate of substrate (Suc-LLVY-AMC) degradation. Following the kinetics of classic irreversible inhibition, the curves intersect the horizontal axis at a position equivalent to the amount of enzyme that it irreversibly inactivated (Figure S2). Consistent with this kinetic analysis, washing experiments confirmed that proteolytic activity was irreversibly abrogated in the presence of palau'amine (Figure 1B). The chymotryptic-like proteolytic activity of the 20S proteasome is completely abrogated following the treatment with an excess of the reversible inhibitor MG-132;[27] however, the proteolytic activity is almost completely restored (Figure 1B) following the removal of drug by excessive washing with reaction buffer (500×). In contrast, treatment of the 20S proteasome, with the irreversible proteasome inhibitor epoxomicin,[34] abrogates proteasome activity both pre- and

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post-washing, indicative of irreversible binding of the drug. Similar to epoxomicin, treatment of 20S proteasome with palau'amine (**1**) resulted in complete abrogation of CT-L proteolytic activity in both pre- and post-washing experiments, indicative of irreversible-type binding (Figure 1B).

Palau'amine Induces Accumulation of Ubiquitinylated Proteins *in Vivo*. Inhibition of the human proteasome results in the accumulation of ubiquitinylated proteins in cells.[35] To investigate whether palau'amine inhibits the proteasome in cells, we examined the accumulation of ubiquitinylated cellular proteins using fluorescence confocal microscopy of HeLa cells following 2 h of incubation with either vehicle (0.1% DMSO) or palau'amine (1.0 μM). As seen in Figure 2, treatment of the cells with vehicle (Figure 2, panel B) versus palau'amine (Figure 2, panel D) resulted in a robust increase in red fluorescence after staining with an Alexa-546 conjugated antibody directed toward ubiquitin. This accumulation of ubiquitinylated proteins is consistent with proteasome inhibition. Panels A and C are DIC images of the cells. A 60× magnification of vehicle (Figure 2, panel E) versus palau'amine (Figure 2, panel F) illustrates the same dramatic accumulation of ubiquitinylated proteins. Cells were stained for ubiquitin (red), and DAPI (blue) was used to visualize the integrity of the DNA within the nucleus. Quantification of the fluorescent intensity using the ubiquitin−Alexa546 conjugate provided a statistically significant increase in accumulation of ubiquitinylated proteins (p = 0.001).

Palau'amine Inhibits the Degradation of IκBα. The anti-inflammatory and anticancer activity of proteasome inhibitors has been linked, in part, to their ability to inhibit the pro-inflammatory antiapoptotic NF-κB signaling pathway.[31,36] The nuclear transcription factor NF-κB is sequestered in the cytoplasm by the inhibitory protein κB, termed IκBα. Activation of the NF-κB pathway by cytokines, such as TNF-α, results in the rapid ubiquitinylation and proteasomal degradation of IκBα, which releases NF-κB for nuclear translocation and gene transcription. Thus, proteasome inhibitors prevent IκBα from degrading and result in an accumulation of cytosolic ubiquitinylated IκB, following TNF-α activation of the NF-κB pathway. To determine if palau'amine prevented the proteasomal degradation of IκBα, we used confocal microscopy to visualize the accumulation of IκBα in the presence and absence of palau'amine (1.0 μM) in HeLa human cervical adenocarcinoma cells. Cells were stained for IκBα conjugates (red), and DAPI (blue) was used to visualize the DNA. In vehicle-treated cells, IκBα is distributed throughout the cytoplasm (Figure 3, panel A), whereas upon stimulation with TNFα, IκBα is degraded and the fluorescent signal (red) is significantly decreased (Figure 3, panel B). Cells treated with palau'amine retained a robust fluorescent signal (Figure 3, panel C). Treatment of the cells with palau'amine prior to TNF-α stimulation also retained robust fluorescent signal (Figure 3, panel D), and this retention of the fluorescence indicates that palau'amine inhibited the proteolytic degradation of IκBα by the 26S proteasome. Quantification of the fluorescent intensity using the IκBα−Alexa568 conjugate illustrates that the prevention of IκBα degradation by palau'amine is statistically significant (p = 0.005), compared to its respective TNFα/vehicle control (Figure 3E).

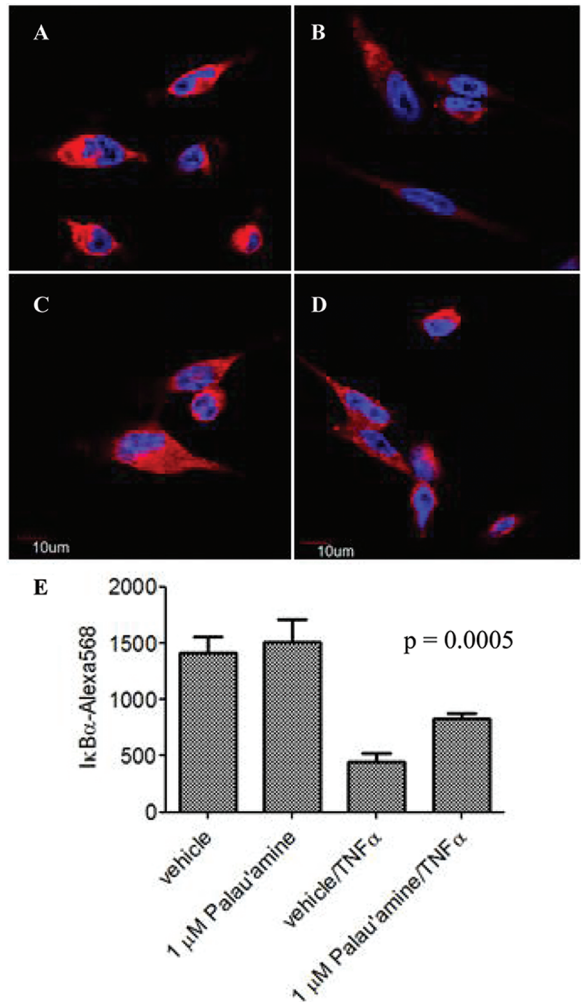

Figure 3. Palau'amine inhibits the degradation of IκBα in HeLa cells. Cells were treated with vehicle (A and B) or 1 μM palau'amine (C and D) for 30 min. Cells were stimulated with 20 ng/mL TNFα (B and D) for 30 min. The cells were fixed and stained for IκBα conjugates (red), and DAPI (blue) was used to visualize the DNA. (E) Quantification of fluorescent intensity of the IκBα−Alexa 568 conjugate. The p-value signifies the statistical significance between the vehicle/TNFα and palau'amine/TNFα.

CONCLUSION

We report herein that the oroidin-derived alkaloids palau'amine (**1**), dibromophakellin (**2**), and dibromophakellstatin (**3**) inhibit the proteolytic activity of the human 20S proteasome as well as the ^{i}20S immunoproteasome catalytic core. Palau'amine was further investigated for its cellular mechanism using confocal microscopy and found to prevent the degradation of ubiquitinylated proteins, including IκBα. The in vitro activity of these agents against the purified human proteasome was in the low micromolar IC_{50} values (IC_{50} 4−7 μM, Table 1), whereas a robust modulation of protein proteolysis can be observed at 1.0 μM concentrations in cell culture. A likely explanation for the robust cellular activity of palau'amine may be due to its inherent instability at the pH of the in vitro assays (pH 7.5), compared to the cellular environment (pH 7.1−7.2). The cellular potency of the compounds corresponds well to its reported cellular activities and may be indicative of the potential mechanism by which these agents exhibit their exciting biological properties.

EXPERIMENTAL SECTION

Materials. (±)- and (−)-Palau'amine (**1**) were obtained from Prof. P. Baran, as an TFA salt. (±)-Dibromophakellstatin (**3**) was obtained

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from Prof. K. Feldman. (±)-Dibromophakellin (**2**) and **4** were prepared in the Tepe laboratory.

20S Proteasomal Activity Measurement. The fluorogenic substrates Suc-LLVY-AMC (substrate for CT-L activity), Boc-LRR-AMC (substrate for T-L activity), and Z- LLE-AMC (substrate for Casp-L activity) were used to measure proteasome activity.[32] Assays were carried out in black, clear-bottom 96-well plates in a 200 μL reaction volume containing 1 nM purified human 20S proteasome in 50 mM Tris-HCL pH 7.5 and 0.03% SDS containing fluorogenic substrate (at concentrations corresponding to their respective K_m value) at 37 °C. The rate of cleavage of fluorogenic peptide substrates was determined by monitoring the fluorescence of released amino-methylcoumarin using a SpectraMax M5e multiwall plate reader at an excitation wavelength of 380 nm and emission wavelength of 460 nm. Fluorescence was measured every minute over a period of 30 min, and the maximum increase in fluorescence per minute was used to calculate specific activities of each sample.

Determination of Reversibility. A membrane filter "washout" assay was used to determine the reversibility of the test agents. Human 20S proteasome (1 nM) was treated with either vehicle, 1 μM MG-132, 1 μM epoxomicin, or 10 μM palau'amine, and the reaction was assayed for CT-L activity as described above. To determine reversibility, the reactions were washed through Amicon Ultracel 10K centrifugal filter units with 500 volumes of reaction buffer. The resulting filter retentates were assayed for CT-L activity and compared to the prewashed activity. Reversibility was also monitored using enzyme kinetics. Palau'amine was added at various concentrations to either 1, 0.5, 0.25, or 0.125 nM activated human 20S in 50 mM Tris-HCL pH 7.5, 0.03% SDS. The kinetics of the reactions was followed using the substrate Suc-LLVY-AMC at 50 μM (the previously determined K_m) on a black, clear-bottom 96-well plate (λ_{ex} 380, λ_{em} 440). The linear portions of the curve were used to determine V_{max}, and reversibility was determined by plotting V_{max} versus $[E]$. For a reversible inhibitor, the curve will have a smaller slope than the control (vehicle) curve and will go through the origin, while the curve for an irreversible inhibitor will intersect the horizontal axis at a position equivalent to the amount of enzyme that it irreversibly inactivated.

Confocal Microscopy. HeLa human cervical adenocarcinoma cells were grown in MEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. HeLa cells were grown on glass coverslips and treated with vehicle (0.1% DMSO) or 1.0 μM palau'amine. After pretreatment with compounds, the cells were stimulated with 20 ng/mL TNFα (30 min for IκBα and 2 h for ubiquitin experiments), while the control cells were left unstimulated. Cells were then fixed with 4% paraformaldehyde in PBS, permeabilized in 0.5% Triton in PBS, and preincubated for 1 h in blocking buffer (5% BSA, 0.05% Tween-20 in PBS). The cells were stained overnight with primary antibody at 4 °C (1:250 of either rabbit anti-ubiquitin (P4D1) or mouse anti-IκBα (L35A5) in blocking buffer) and for 1 h with Alexa546-labeled secondary antibody (1:1000, Invitrogen). Coverslips were mounted with Fluorogel containing DAPI (4,6-diamidino-2-phenylindole; 1 mg/mL). Cells were imaged using an Olympus FV1000 scanning confocal microscope using the one-way XY scan mode.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Full experimental protocols, graphs, and error analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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