# Palau'amine and related oroidin-alkaloids dibromophakellin and dibromophakellstatin inhibit the human 20S proteasome

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Supplemental Information:

Contents

**20S Proteasomal activity measurement**

The fluorogenic substrates Suc-LLVY-AMC, Z-ARR-AMC, and Z-LLE-AMC were used to measure CT-L, T-L and Casp-L proteasome activities respectively. Assays were carried out in black, clear bottom 96 well plates in a 200 μL reaction volume containing 1 nM of purified human 20S proteasome in 50 mM Tris-HCL pH 7.5 and 0.03% SDS (for CT-L and Casp-L) or 0.01% triton X-100 in 50 mM Tris-HCl pH 7.5 (for T-L), containing 50 μM fluorogenic substrate at 37° C. In these experiments, the drug and enzyme are combined and pre-incubated for 30 min, after which the substrate is added and the rates of hydrolysis are monitored over time The rate of cleavage of the fluorogenic peptide substrates was determined by monitoring the fluorescence of released aminomethylcoumarin using a SpectraMax M5e multiwall plate reader at an excitation wavelength of 380 nm and emission wavelength of 460 nm. The rates of hydrolysis were monitored by fluorescence increase at 37° C over 30 minutes and the linear portion of the curve was used to determine the rate of hydrolysis. The rates were used to determine the dose response ($IC_{50}$).

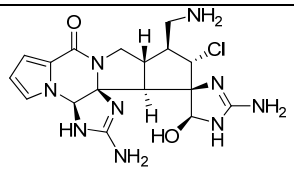
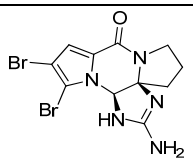
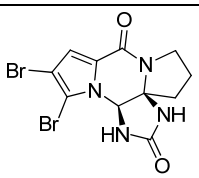
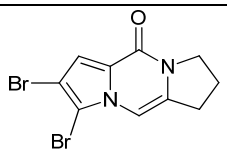
| compound |  (±)-**1** (-)-**1** |  **2** |  **3** |  **4** |
|---|---|---|---|---|
| Experiment 1 | 4.78<br>3.18 | 20.20 | 11.27 | >100 |
| Experiment 2 | 5.88<br>2.41 | 30.80 | 11.67 | >100 |
| Experiment 3 | 7.45<br>1.88 | 24.20 | 12.86 | >100 |
| Experiment 4 | 3.93<br>nt | 25.93 | 11.71 | >100 |
| average | **5.51±1.52**<br>**2.49±0.65** | **25.28 ±4.39** | **11.88 ±0.68** | **>100** |

**Table S1.** Inhibition of the chymotryptic-like (CT-L) activity of purified human 20S proteasome and CT-L activity by oroidin-derived alkaloids: racemic palau'amine ((±)-**1**), (-)-palau'amine ((-)-**1**), *rac*-dibromophakellin (**2**) and *rac*-dibromophakellstatin (**3**) and synthetic precursor **4.**

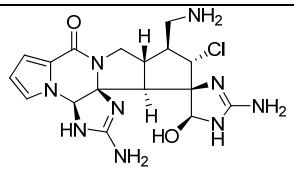
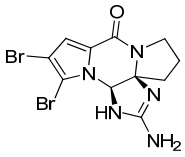
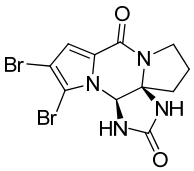
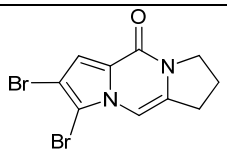
| compound |  (±)-**1** (-)-**1** |  **2** |  **3** |  **4** |
|---|---|---|---|---|
| Experiment 1 | >100<br>>100 | >100 | >100 | >100 |
| Experiment 2 | >100<br>>100 | >100 | >100 | >100 |
| average | **>100** | **>100** | **>100** | **>100** |
| | | | | |

**Table S2.** Inhibition of the Tryptic-like (T-L) activity of purified human 20S proteasome by oroidin-derived alkaloids: racemic palau'amine ((±)-**1**), (-)-palau'amine ((-)-**1**), *rac*-dibromophakellin (**2**) and *rac*-dibromophakellstatin (**3**) and synthetic precursor **4.**

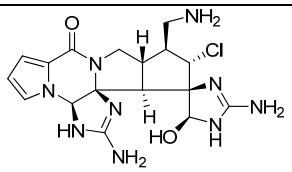
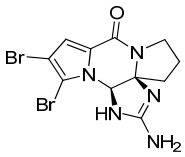
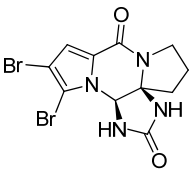
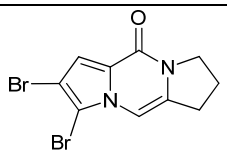
| compound |  (±)-**1** (-)-**1** |  **2** |  **3** |  **4** |
|---|---|---|---|---|
| Experiment 1 | 2.68<br>2.14 | 28.06 | 16.05 | >100 |
| Experiment 2 | 3.22<br>0.96 | 20.16 | 17.50 | >100 |
| Experiment 3 | --- | 32.96 | 15.07 | >100 |
| average | **2.95±0.38**<br>**1.55±0.83** | **27.06±6.45** | **16.21±0.1.22** | **>100** |

**Table S3.** Inhibition of the Caspase-like (Casp-L) activity of purified human 20S proteasome by oroidin-derived alkaloids: racemic palau'amine ((±)-**1**), (-)-palau'amine ((-)-**1**), *rac*-dibromophakellin (**2**) and *rac*-dibromophakellstatin (**3**) and synthetic precursor **4.**

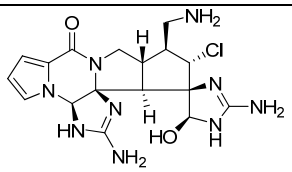
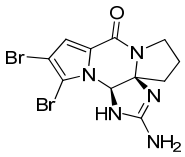
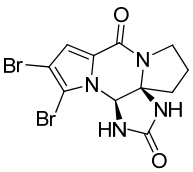
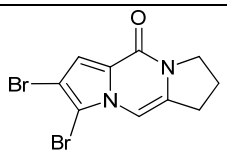
| compound |  (±)-**1** (-)-**1** |  **2** |  **3** |  **4** |
|---|---|---|---|---|
| Experiment 1 | 4.23<br>2.32 | 18.7 | 4.6 | >100 |
| Experiment 2 | 3.73<br>2.30 | --- | 7.77<br>6.2 | >100 |
| average | **3.98±0.35**<br>**2.31±0.01** | **18.7** | **6.19±0.1.6** | **>100** |
| | | | | |

**Table S4.** Inhibition of the chymotryptic-like (CT-L) activity of purified human $^{i}$20S immunoproteasome by oroidin-derived alkaloids: racemic palau'amine (rac **1**) *rac*-dibromophakellin (**2**) and *rac*-dibromophakellstatin (**3**) and synthetic precursor **4.**

**Stability of Palau'amine in 50 mM Tris-HCL pH 7.5 and pH 8.0**

**Method**: Vehicle, or 20, 10, 5, 2.5, 1.25, 0.63 or 0.31 μM agent was incubated in the corresponding buffer (50 mM Tris-HCL pH 7.5 or pH 8.) for the indicated times at 37°C. Upon incubation, human 20S proteasome was added to 1 nM and activated with SDS (final concentration 0.03%). The fluorogenic substrate (5 μM), Suc-LLVY-AMC, was used to measure CT-L proteasome activity. The rate of cleavage of fluorogenic peptide substrate was determined by monitoring the fluorescence of released aminomethylcoumarin 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 minutes and the maximum increase in fluorescence per minute was used to calculate specific activities of each sample.

**pH 7.5**

| Agent | 0 minutes | 20 minutes | 60 minutes |
|---|---|---|---|
| Control proteasome inhibitor TCH-013 | 2.5 μM | 2.3 μM | 2.9 μM |
| Palau'amine (rac) | 5.9 μM | >100 μM | >100 μM |

**pH 8.0**

| Agent | 0 minutes | 20 minutes | 60 minutes |
|---|---|---|---|
| Palau'amine (rac) | 15.1 μM | >100 μM | >100 μM |

**Figure S1. Stability of palau'amine towards assay conditions:** The activity of palau'amine was evaluated at pH 7.5 and pH 8.0 at various times **prior** to exposure of the compound to the 20S proteasome. In the previous assays, palau'amine was exposed to the 20S proteasome, which is different from the experiment below. Control compounds include structurally unrelated proteasome inhibitor (and pH stable) TCH-013.

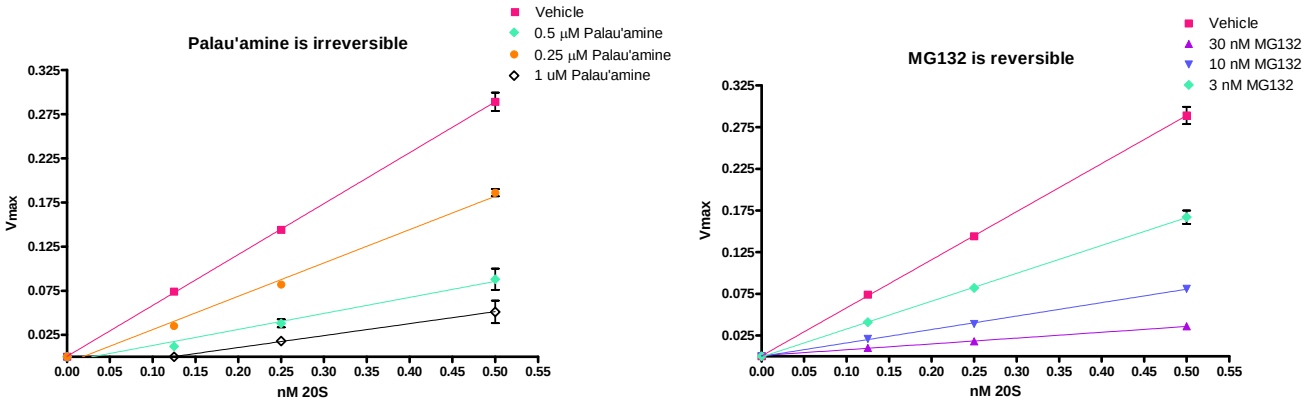



**Figure S2.** Kinetics for inhibition of CT-L activity using varying concentrations of palau'amine (**1**) and reversible inhibitor MG-132.