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Thiasyrbactins Induce Cell Death *via* Proteasome Inhibition in Multiple Myeloma Cells

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Running title: Thiasyrbactins Inhibit Proteasomal Activity in MM Cells

(Experimental Study)

Key Words: Proteasome inhibitors, immunoproteasome, NAM, thiasyrbactin, caspase-like, trypsin-like, chymotrypsin-like activity, multiple myeloma.

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Abstract. Background/Aim: Proteasome inhibition is a validated therapeutic strategy for the treatment of refractory and relapsed multiple myeloma (MM) and mantle cell lymphoma. We previously showed that *in vitro*, thiasyrbactins (NAM compounds) are inhibitors with an affinity for the trypsin-like (T-L, β 2) site of the constitutive proteasome, and more profoundly on the T-L site of the immunoproteasome. Materials and Methods: In this study, the biological activity of three NAM compounds was evaluated on four hematologic cell lines of plasma immune cells. Using four MM cell lines (ARD, U266, MM1R, and MM1S) we assessed the effect of three NAM compounds (NAM-93, NAM-95, and NAM-105) on cell viability as well as cell-based proteasomal activities, and determined the EC_{50} and Ki_{50} values. Results: MM cells were most sensitive to NAM-93 with EC_{50} values $<0.75 \mu\text{M}$ after 48 h of treatment. NAM-105 had a similar profile in most of the MM cells with EC_{50} values ranging between 0.42 and 3.02 μM . The level of inhibition of the proteasome T-L sub-catalytic activity in actively-growing MM cells was similar for NAM-93 and NAM-105. However, in each cell line, NAM-93 was more effective than NAM-105 at inhibiting overall trypsin-like sub-catalytic activity while NAM-105 was typically more effective at inhibiting overall chymotrypsin-like (CT-L, β 5) sub-catalytic activity. Conclusion: These results show for the first time the proteasome-targeted biological activity of thiasyrbactins in MM tumor cells.

The proteasome is involved in a vast number of functions within normal cells. It is also heavily involved in the pathologic progress of several disease states including hematological cancers, autoimmune disorders, and inflammatory diseases. The active core of the proteasome consists of 2 protein rings consisted of 7 subunits ($\beta 1 - \beta 7$). Three of these subunits perform a catalytic step in degrading proteins. They are denoted $\beta 1$ (caspase-like, C-L), $\beta 2$ (trypsin-like, T-L), and $\beta 5$ (chymotrypsin-like, CT-L). The activity of the subunits varies depending on the proteasome isoform. In addition to the constitutive proteasome, other isoforms include the immunoproteasome, which is induced in inflammation and *via* the action of some cytokines (*e.g.*, interferon γ) (1-5).

Proteasome inhibition is a validated therapeutic strategy against several forms of cancer (3,6). Indeed, FDA-approved proteasome inhibitors, bortezomib (BTZ), ixazomib (IXA), and carfilzomib (CAR) have had clinical success in hematological cancers such as multiple myeloma (MM) and mantle cell lymphoma (7-10). Bortezomib and ixazomib are part of the boronate class, while carfilzomib is part of the epoxyketone class. These proteasome inhibitors effectively target the constitutive proteasome. However, the immunoproteasome is the target of next-generation proteasome inhibitors particularly in autoimmune disorders and inflammatory diseases (11-13).

The syrbactins are a new class of natural products-based proteasome inhibitors that selectively and covalently bind to the catalytic Thr1 residue of the proteasome by a novel mechanism (14). Syrbactins include glidobactins (15-19), cepafungins (20,21), and syringolins (1,3,14,22,23). Although glidobactins and cepafungins have been described many years ago, the mode of action (proteasome inhibition) remained elusive until recently (14). Our discovery spurred multiple investigations into elucidating the total synthesis of the natural products Syringolin A/B (SylA/B) (24-26). This prompted the design of a number of syrbactin-inspired analogs with improved biological activity and anti-proliferative potency (26-35). TIR-203 was inspired by SylB and inhibits proteasome activity and growth of multiple myeloma and neuroblastoma (NB) cell lines (35). The syrbactin structural analog TIR-199 was the first syrbactin to show efficacy in an *in vivo* cancer model (29), and both TIR-203 and TIR-199 were also investigated by the National Cancer Institute, Developmental Therapeutics Program (NCI-

DTP). In an attempt to make improvements to the TIR-199 molecule, the NAM family of analogs was designed (30). NAM-105 was inspired by TIR-199, as most of its structure is similar except for the isolated alkene. NAM-93 and NAM-95 were specifically designed to improve solubility. Recently, we reported that these thiasyrbactins preferentially inhibited the T-L (β_2) subunit of the immunoproteasome using an *in vitro* proteasomal activity assay that relies on purified immunoproteasomes (30). In this paper, we present evidence that these thiasyrbactins pass the cell membrane and induce cell death of actively dividing MM cells *via* inhibition of the proteasome.

Materials and Methods

Chemical reagents. Thiasyrbactins (NAM-93, NAM-95, and NAM-105) were synthesized as reported (30) and solubilized in DMSO solution (Figure 1). Bortezomib (Velcade[®]) was purchased from LC Laboratories (Woburn, MA, USA). All drug solutions were prepared at 10 mM in DMSO (NAMs and bortezomib), sterile-filtered, and stored frozen at -80°C . At the beginning of each experiment, aliquots were thawed and diluted to the final concentration.

Mammalian cell cultures and reagents. Authenticated human MM cell lines were obtained from certified suppliers between 2014 and 2016. ARD (Van Andel Research Institute, Grand Rapids, MI, USA); U266 (Van Andel Research Institute); MM1R (ATCC, Manassas, VA, USA), MM1S (ATCC). MM1R cell line derived from a patient who had become resistant to steroid-based therapy, dexamethasone. All cell lines were maintained in Roswell Parke Memorial Institute (RPMI) 1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and supplemented with penicillin (100 U/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 and plated 24 h before drug treatment. Control cells were treated with 1% DMSO in culture media, equivalent to the maximum amount of DMSO present at the highest doses of drug.

Cell viability assay. The RealTime-Glo Assay (Promega, Madison, WI, USA) was used to determine the viability of cancer cells after 24 and 48 h treatment. Cells were seeded 10,000 cells/well in solid white 96-well plates with media containing RealTime-Glo reagents as specified by the manufacturer's instructions. Luminescence was measured with a Biotek Synergy microplate reader at 0 and 24 h after seeding and again after 24 h and 48 h of drug treatment. Data were expressed as relative light units and normalized to the well with the highest concentration of DMSO.

Cell-based proteasome activity assay. The cell culture-based proteasome-Glo inhibition assay (Promega) was performed as previously described (29). Cells were seeded in solid white 96-well plates 24 h prior to treatment. Cells were then treated with 0 – 10 μ M of indicated drug for 24 h. Cells were incubated for 15 min with the proteasome GloTM reagents according to the manufacturer's instructions and the inhibition of the proteasomal sub-catalytic activities (C-L, T-L, CT-L) were measured by addition of luminogenic substrates Z-nLPnLD-aminoluciferin, Z-LRR-aminoluciferin, and Suc-LLVY-aminoluciferin, respectively.

Statistical analyses. GraphPad Prism v7.04 was used to generate cell viability (EC_{50}) curves and proteasome inhibition (K_i) curves. The EC_{50} values were determined from a non-linear regression fit as log(inhibitor) vs. response, variable slope (four parameters). K_i values were determined from a non-linear regression fit as [inhibitor] vs. normalized response.

Results

NAM compounds have distinct cell viability profiles. Previous studies from our group have demonstrated the extent of constitutive proteasome and immunoproteasome inhibition by thiasyrbactins (NAMs) using an *in vitro*-based proteasomal activity assay. The tested NAM compounds predominantly inhibited the CT-L and T-L activities of the constitutive proteasome, but potently and selectively inhibited

the T-L activity of the immunoproteasome, with little effect on the CT-L and C-L sub-catalytic sites (30).

In this study, the effects of NAM-93, NAM-95, and NAM-105 were firstly assessed on the viability of actively-dividing MM tumor cell lines ARD, U266, MM1R, and MM1S. As a positive control, bortezomib (BTZ) was included at one single concentration (0.05 μM). Even though the three NAM compounds have similar *in vitro* effects on the sub-catalytic activities of the constitutive proteasome and immunoproteasome, their cell viability profiles were markedly different. Figure 2 shows that the MM cells were most sensitive to NAM-93 after 24 and 48 h with EC_{50} values $<0.75 \mu\text{M}$ (determined at 48 h). Of note, the dexamethasone-resistant cell line MM1R and the dexamethasone-sensitive cell line MM1S responded in a similar manner to NAM-93. NAM-105 demonstrated a comparable profile in most MM cells with EC_{50} values ranging between 0.42 – 3.02 μM . In contrast, NAM-95 showed significantly higher EC_{50} values, from 16.93 to $>30 \mu\text{M}$. EC_{50} values are displayed in Table I.

NAM compounds have distinct proteasome inhibition profiles. To assess if the three NAM compounds have the same proteasome inhibition profile in cells as previously observed in our *in vitro* experiments (30), a cell-based proteasome activity assay was used to measure the three sub-catalytic activities in four MM cell lines. Figure 3 shows the dose-dependent inhibitory effects of NAM-93, NAM-95, and NAM-105 on the C-L, T-L, and CT-L sub-catalytic activities of the proteasome. The Ki_{50} numeric values for the overall (constitutive and immunoproteasome) sub-catalytic activities, after a 24-h incubation with NAM compounds are displayed in Table II.

NAM-93 inhibited all three active sites (C-L, T-L, and CT-L) in four MM cell lines. NAM-93 was the least selective for the C-L site (Ki_{50} values 1.1–5.3 μM) and was most selective in inhibiting the T-L site with Ki_{50} values as low as 0.27 μM –1.0 μM . This is likely due to the fact that the T-L site is the only active site inhibited by NAM-93 in both the constitutive and immunoproteasome isoform. NAM-95 showed selectivity for CT-L site with Ki_{50} values $\sim 7.5 \mu\text{M}$ in two of the four cell lines. NAM-105 inhibited each of the three active sites in all four cell lines and showed specificity for the CT-L site with Ki_{50} values as low as 0.10–1.08 μM .

Despite the similar *in vitro* inhibition profiles for CT-L and T-L sites (30), NAM-95 was the least

effective NAM and inhibited the proteasomal activity with K_{i50} values ranging from 7.5 to over 10 μM in all four cell lines. When comparing the NAM-93 and NAM-105 profiles, C-L inhibition was similar between the four cell lines. However, in each cell line, NAM-93 was more effective than NAM-105 at inhibiting the T-L sub-catalytic activity, while NAM-105 was usually more effective at inhibiting the CT-L sub-catalytic activity.

Discussion

The immunoproteasome is a next-generation target for proteasome inhibitors for hematologic malignancies, autoimmune diseases, and immune disorders (4). NAM compounds have been shown to inhibit the T-L subunit of the immunoproteasome. As these compounds had moderate biological activities against NB cells (30), we here tested the effects of these compounds on four hematologic cell lines of plasma immune cells. In cell viability assessments, the EC_{50} values for NAM-93 and NAM-105 were $<2.08 \mu\text{M}$ across all MM cell lines with sub-micromolar concentrations for NAM-93. NAM-95 had the least effect on cell viability, which was expected as it also had the weakest inhibitory activity of the proteasome.

As the cell-based proteasome activity assay is not able to distinguish between constitutive proteasomes and immunoproteasomes, our results assess overall proteasomal activity and not the contribution of each isoform. However, the MM cell lines do contain both constitutive proteasome and immunoproteasome (36,37), which is likely why the anti-proliferative effect of the three NAM compounds are more pronounced in the MM cells *versus* the NB cells.

Our new results are promising and justify the further exploration of NAM compounds in immune and cancer cells. Our data show for the first time the biological activity of the thiasyrbactins in a hematological malignancy cell culture model. Cells that express higher proportions of immunoproteasome compared to constitutive proteasome may exhibit a marked increase in cell death and proteasomal inhibition. Under these conditions, synergistic effects might be observed in combination with other proteasome inhibitors, chemotherapeutic and antiviral drugs (38,39) or immunomodulatory agents.

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Conflicts of Interest

M.C.P., N.A.B., and A.S.B. are named inventors on a US Patent application concerning the thiasyrbactins. A.S.B is also a named inventor of United States patent (US 8,597,904, December 3, 2013) that relates to pharmaceutical compositions for the treatment of conditions responsive to proteasome inhibition. M.C.P and A.S.B are the founders of Hibiskus Biopharma, Inc (Kalamazoo, MI, USA). No potential conflicts of interest were disclosed by the other Authors.

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Figure 1

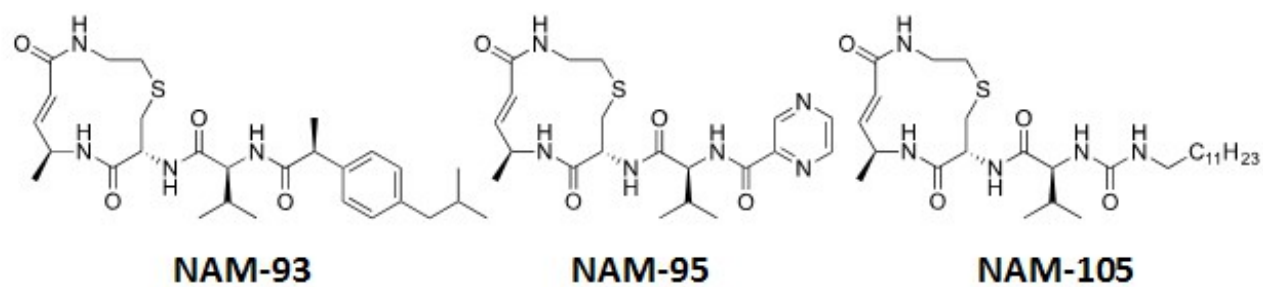


Figure 1. Structures of thiasyrbactins representing novel syrbactin-based proteasome inhibitors. The molecular weights for NAM-93, NAM-95, and NAM-105 are 531, 449, and 554 Da, respectively.

Figure 2

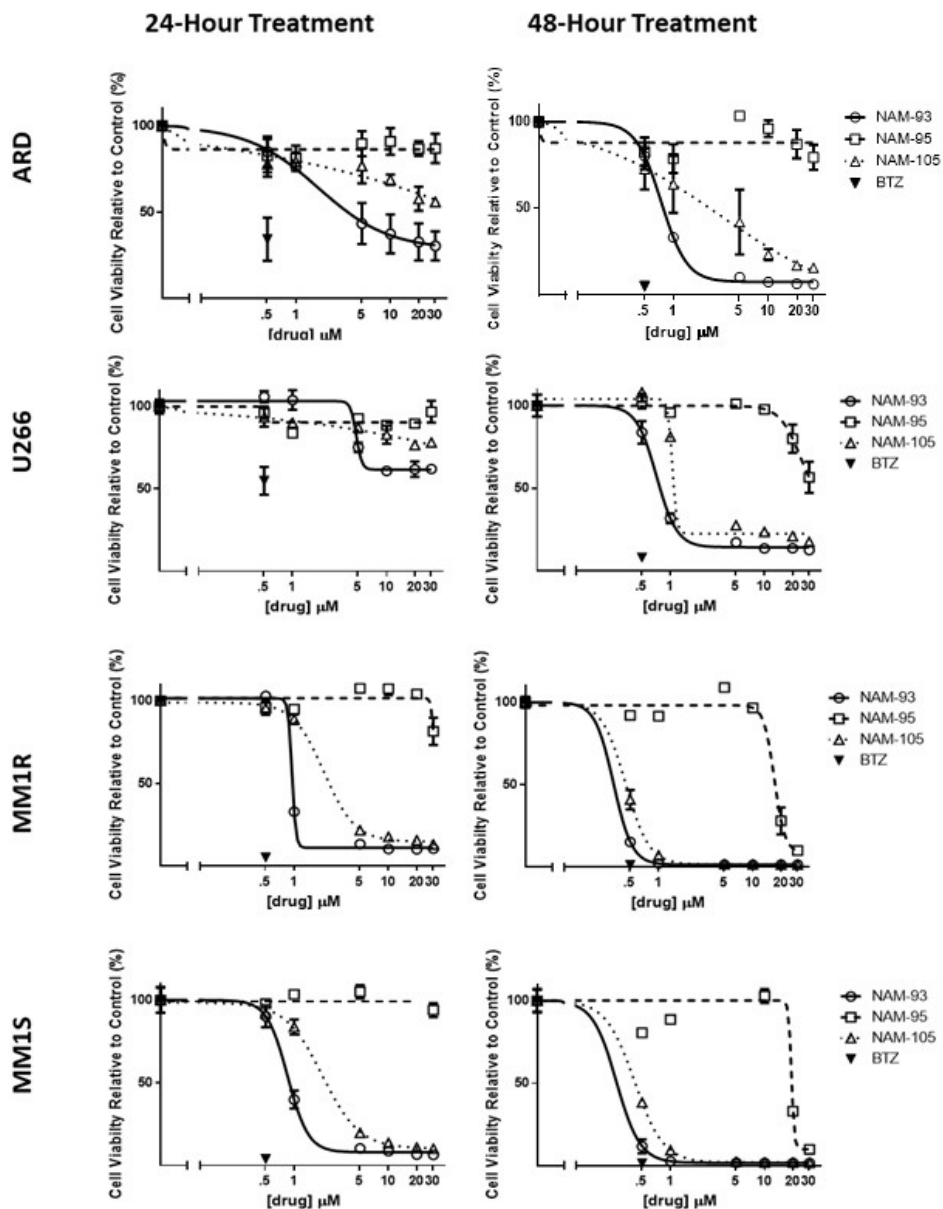


Figure 2. NAM compounds have distinct cell viability profiles. Cell viability was determined at 24 h and 48 h after exposure of multiple myeloma (MM) cell lines, ARD, U266, MM1R, and MM1S to proteasome inhibitors NAM-93, NAM-95, and NAM-105. Bortezomib (BTZ) was used as a positive control. Cell viability was inhibited in a dose-dependent manner. Data was collected from three individual experiments ($n=3$). The EC_{50} values were determined from a non-linear regression fit as $\log(\text{inhibitor})$ vs. response, variable slope (four parameters). See Table I for numeric EC_{50} values.

Figure 3

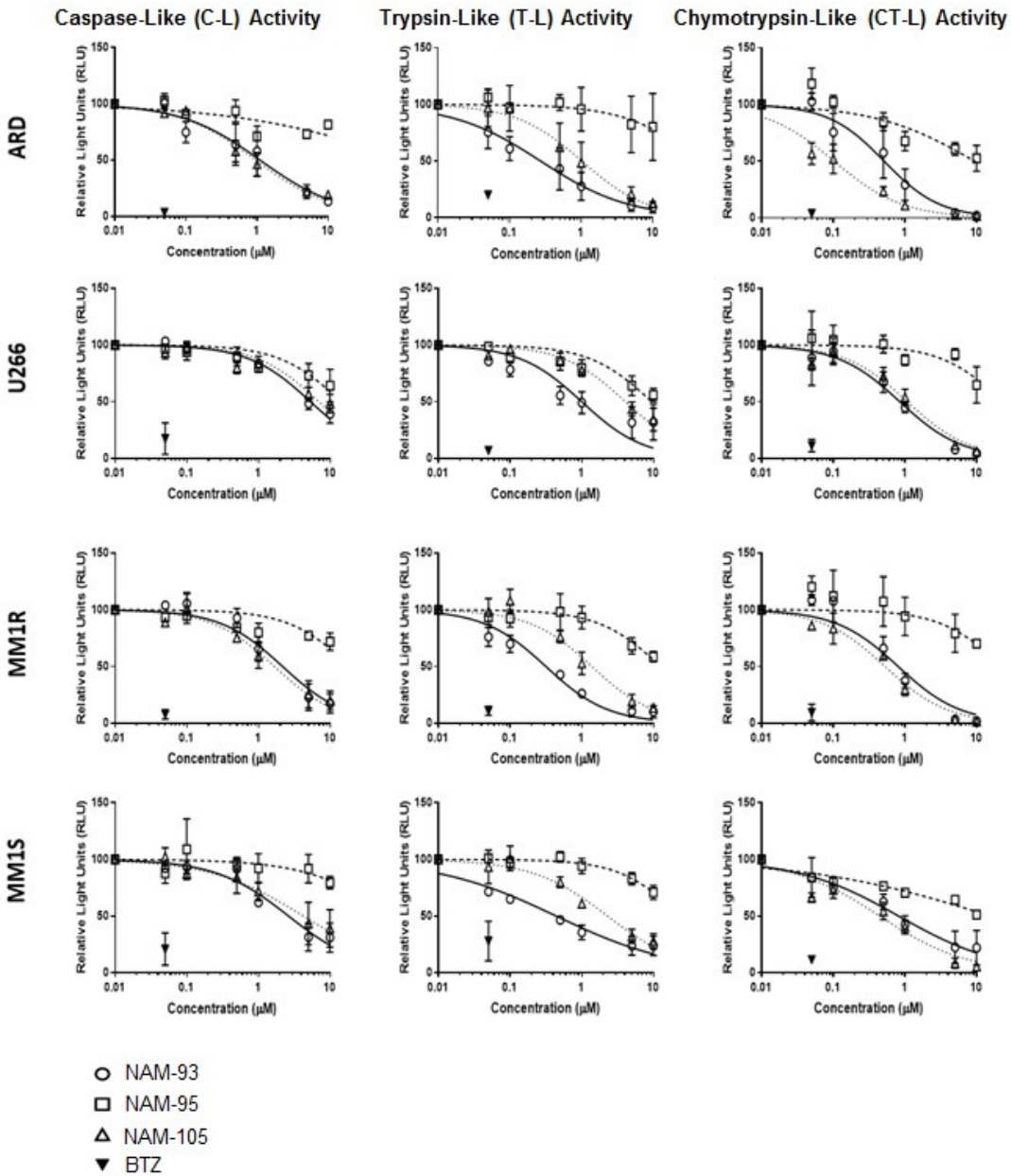


Figure 3. NAM compounds have distinct overall proteasome inhibition profiles. Multiple myeloma (MM) cell lines, ARD, U266, MM.1R, and MM.1S were exposed to proteasome inhibitors NAM-93, NAM-95, and NAM-105. Bortezomib (BTZ) was used as a positive control. Cell-based proteasome activity was inhibited in a dose-dependent manner. Data was collected from three individual experiments ($n=3$). Ki_{50} values were determined from a non-linear regression fit as $[\text{inhibitor}]$ vs. normalized response. See Table II for numeric Ki_{50} values.

Table 1. *NAM syrbactin analog-induced inhibition of cell viability displayed as EC₅₀ (μM) values for 24- and 48-hour treatments. NAM-93 is the most potent analog across multiple myeloma (MM) cell lines. Data calculated from Figure 2.*

24-Hour Cell Viability, EC₅₀ (μM)

	NAM-93	NAM-95	NAM-105
ARD	1.72	>30	>30
U266	>30	>30	>30
MM1R	0.95	>30	2.08
MM1S	0.85	>30	1.99

48-Hour Cell Viability, EC₅₀ (μM)

	NAM-93	NAM-95	NAM-105
ARD	0.75	>30	3.02
U266	0.72	24.31	1.05
MM1R	0.33	16.93	0.44
MM1S	0.27	19.27	0.42

Table II. *NAM syrbactin analog-induced inhibition of cell-based proteasome sub-catalytic activities displayed as K_{i50} (μM) values. Data calculated from Figure 3.*

Cell-Based Proteasome Inhibition, K_{i50} (μM)			
	NAM-93	NAM-95	NAM-105
ARD			
C-L	1.11 \pm 0.32	>10	0.91 \pm 0.14
T-L	0.27 \pm 0.08	>10	1.01 \pm 0.33
CT-L	0.50 \pm 0.14	7.5 \pm 1.9	0.10 \pm 0.02
U266			
C-L	5.31 \pm 0.64	>10	6.99 \pm 1.15
T-L	1.00 \pm 0.29	9.49 \pm 1.79	4.07 \pm 0.51
CT-L	0.85 \pm 0.16	>10	1.08 \pm 0.16
MM1R			
C-L	2.17 \pm 0.48	>10	1.67 \pm 0.35
T-L	0.32 \pm 0.04	>10	1.38 \pm 0.25
CT-L	0.80 \pm 0.16	>10	0.53 \pm 0.07
MM1S			
C-L	2.59 \pm 0.56	>10	3.98 \pm 1.14
T-L	0.42 \pm 0.10	>10	2.22 \pm 0.39
CT-L	0.80 \pm 0.22	7.51 \pm 1.68	0.50 \pm 0.09