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Muli, Christine
Ali, Eslam
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Discovery of a Non-Covalent Ligand for Rpn-13, a Therapeutic Target for Hematological Cancers

Cody A. Loy[a], **Christine S. Muli**[a], **Eslam M. H. Ali**[a], **Dan Xie**[a], **Mostafa H. Ahmed**[b], **Carol Beth Post**[a], **Darci J. Trader**[a,*]

[a]Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, 575 West Stadium Avenue, West Lafayette, Indiana 47907, United States

[b]Atomwise Inc., San Francisco, California

Abstract

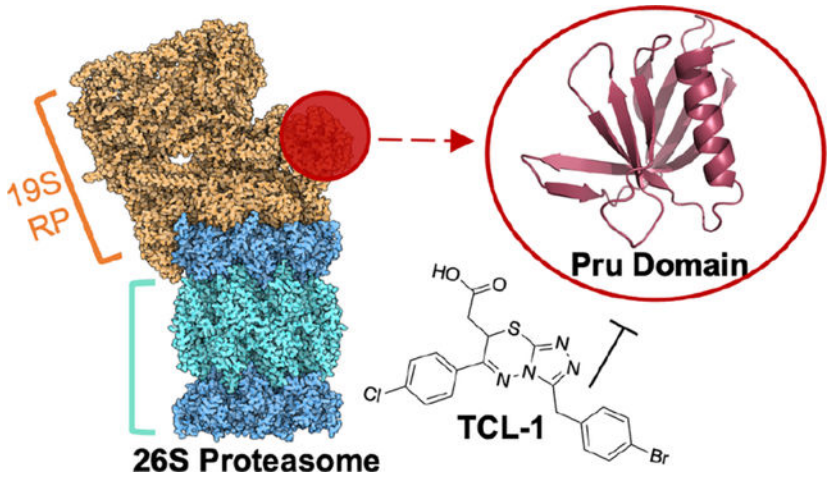
The ubiquitin-proteasome system serves as the major proteolytic degradation pathway in eukaryotic cells. Many inhibitors that covalently bind to the proteasome's active sites have been developed for hematological cancers, but resistance can arise in patients. To overcome limitations of active-site proteasome inhibitors, we and others have focused on developing ligands that target subunits on the 19S regulatory particle (19S RP). One such 19S RP subunit, Rpn-13, is a ubiquitin receptor required for hematological cancers to rapidly degrade proteins to avoid apoptosis. Reported Rpn-13 inhibitors covalently bind to the Rpn-13's Pru domain and have been effective anti-hematological cancer agents. Here, we describe the discovery of TCL-1, a non-covalent binder to the Pru domain. Optimization of TCL-1's carboxylate group to an ester increases its cytotoxicity in hematological cancer cell lines. Altogether, our data provides a new scaffold for future medicinal chemistry optimization to target Rpn-13 therapeutically.

Graphical Abstract

*corresponding author: dtrader@purdue.edu.

Conflict of interest

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



Keywords

Rpn-13; proteasome; hematological cancer

The proteasome plays a key role in the degradation of ~80% of proteins in eukaryotic cells and is involved in the regulation and maintenance of many cellular functions.[1] The proteasome exists in many isoforms with varying functions. The 20S core particle (20S CP) comprises of four heptameric rings (two rings of ß proteins and two rings of α proteins[2,3]) that can degrade intrinsically disordered and misfolded proteins through a ubiquitin-independent process.[4,5] When capped with the 19S regulatory particle (RP), the proteasome is capable of degrading proteins that have been tagged with polyubiquitin.[6,7]

Targeting the 20S proteasome with small molecule inhibitors has become an invaluable tool for the treatment of diseases with high proteasomal dependence, such as multiple myeloma (MM).[8] Bortezomib (Velcade®) has been the best-in-class treatment for MM for many years, and more recent inhibitors have improved upon off-target toxicities and pharmacokinetic parameters.[9,10] Unfortunately, Bortezomib and other 20S CP inhibitors, inevitably develop resistance and molecules with new mechanisms of inhibition are required. Targeting the recognition or processing of a ubiquitinated protein by the 19S RP rather than the proteolytic function of the 20S CP[11,12] has been identified as an alternative target that can minimize the off-target toxicity and resistance seen with current treatment. (Figure 1).[13–15] Rpn-13, a ubiquitin receptor on the 19S RP, is a potential proteasome target as it is overexpressed in several cancers, including MM, pancreatic, ovarian, and colorectal.[16,17] This may be due to a stress induced response to an abundance of misfolded proteins that the proteasome must degrade to prevent apoptosis of the cancer cell. Rpn-13 inhibitors have been developed such as RA190 and XL5, both of which bind covalently at cysteine 88 of RPN-13 via Michael addition[18,20]. KDT-11, a reversible peptoid binder has also been discovered to bind to Rpn-13 and is selectively toxic to multiple myeloma cells. (Figure 2).[19] In this study, we report a non-covalent inhibitor of the Pru (Pleckstrin-like receptor for ubiquitin) domain of Rpn-13 (TCL-1). TCL-1 is shown to bind to the same surface as Rpn-2, which is a Rpn-13 binding partner within the 19S RP, with modest affinity (K_D = 26 μM). TCL-1's structure

is much more drug-like than KDT-11 and it is highly likely that scaffold modifications can increase its binding affinity.[19]

Screening for binders of the Pru domain

To begin a study to discover a non-covalent binder at the Rpn-2/Rpn-13 binding interface, we applied for the Atomwise AIMS award program (AIMS: A19–381). The virtual screen was carried out using the AtomNet ®neural network for structure-based drug design.[22,23] A single global AtomNet model was deployed to predict the binding affinity of small molecules to a target protein. The model was trained with experimental K_i, K_d, and the half maximal inhibitory concentration (IC_{50}) values of several million small molecules and protein structures spanning several thousand different proteins, curated from public databases and proprietary sources. Because the AtomNet technology was a global model, it could be applied to novel binding sites with no known ligands, a prerequisite to most target-specific machine-learning models (see Supporting Information for detailed procedure). Compounds proposed by the virtual docking to bind at the Rpn-2-Rpn-13 interface of the Pru domain were next validated experimentally for their ability to bind. We screened the potential binders by differential scanning fluorescence in a thermal shift assay that monitors the ability of the molecules to shift the melting temperature of the Pru domain of Rpn-13.[24,25] Briefly, the Pru domain of Rpn-13 was combined with a small molecule and a hydrophobic dye. As the mixture is heated over a range of temperatures (25 – 90 °C) in a stepwise manner, the protein will begin to unfold. As the protein denatures, the hydrophobic dye can bind to the protein's unfolded hydrophobic core, generating a fluorescent signal. From these measurements, "stabilizers" (positive shift in melting temperature) or "destabilizers" (negative shift in melting temperature) are discovered.[26] We validated this screening method using two peptides described by the Hill group that mimic the binding interaction of Rpn-13 with the surface of Rpn-2.[27,28] Both the wild-type peptide and a mutant version (P945A) were tested at two concentrations to determine if we could see a shift in melting temperature (Figure S2). The shift in melting temperature using the wild-type peptide was the largest, which reflects its better K_i value as reported by the Hill group (WT K_i ~17 nM; P945A K_i ~1.4 μM).[27] This control experiment highlighted we would be able to detect both nanomolar and micromolar binders to Rpn-13 using this method.

Potential binding molecules were dissolved in DMSO and tested at 25 and 50 μM to monitor changes to melting temperature (Figure S4). We initially screened 96 molecules in singlet and then validated the top ten stabilizers/destabilizers in triplicate (Figure S5). The compound that had the greatest change on Pru's melting temperature in triplicate was selected as our compound of interest (TCL-1, Figure 3A). TCL-1 (50 μM) decreased the T_m of the Pru domain by ~3 °C (Figure 3B). Encouraged by these results, a second set of molecules from Atomwise was chosen based on similarity to the TCL-1 scaffold. The second screening campaign was performed using the methods described above. However, no molecule was able to successfully validate in triplicate other than TCL-1 (Figure S6).

We next tested TCL-1's ability to affect the T_m of the Pru domain of Rpn-13 in a dose-dependent manner. To accomplish this, a range of concentrations of TCL-1 (0–100 μM)

were added to 20 μM Pru domain and any change in T_m was determined (Figure 3C). These results show that starting at 25 μM of TCL-1, Pru's T_m begins to decrease. As a negative control, we included molecule G7, whose structure was similar to TCL-1 in that the heterocycle core was the same and contained a carboxylic acid (Figure S7), and no change in T_m was observed, as desired.

2D Protein HSQC to Determine Binding Interaction

We utilized 2D NMR to characterize the binding interaction of TCL-1 with the Pru domain by chemical shift perturbations (CSPs). The known structure of the Pru domain and chemical shift assignments for a Pru-ligand complex facilitated NMR studies of the Pru domain in the presence of TCl-1. [29,30] ^{15}N-labeled Pru domain was expressed/purified and concentrated to 200 μM for TROSY ^{15}N-HSQC analysis. TCL-1 was added to the sample in a ligand/protein (L/P) molar ratio of 0, 0.2, 0.4, 0.6, 0.8, and 1, ensuring the protein or ligand did not precipitate. Overlay of spectra measured at each titration point shows observable shifts in the resonant frequencies (Figure 4A). The CSPs were quantified for each residue (Figure 4B). The protein was not fully saturated at 200 μM of TCL-1 as higher concentrations of ligand resulted in loss of Pru domain signal from TCL-1's protein destabilization effects. However, peaks with relatively larger CSPs were still identified for several residues (Figure 4A and 4B). Line shape analysis performed on two selected peaks (G91 and M31) using the web-based tool NmrLineGuru estimate resulted in a K_d value of ~26 μM. (Figure S17).[31] *These results confirm that TCL-1 binds to the Pru domain at the Rpn-2 docking site, which was the target region from the Atomwise docking process.*

Activity of TCL-1 against Cancer Cell Lines

Encouraged by the NMR studies, we were interested to see if TCL-1 would be selectively toxic to hematological cancer cells, due to their high proteasomal activity for survival. Concerned with the permeability of TCL-1's carboxylic acid group, we synthesized a derivative of TCL-1 that incorporated an ester group to aid in cellular permeability, named TCL-Ester (Scheme S1). Before testing the TCL-Ester in cells, we wanted to ensure modifying the carboxylic acid would not affect the binding to the Pru domain. In order to assess this we synthesized a fluorescent derivative (FL-TCL-1) that incorporated a fluorescein tag that we could perform fluorescence polarization with (Scheme S2). Modifying the carboxylic acid of TCL-1 to the FL-TCL-1 derivative did not impact binding to the Pru domain (Figure S18). The ester was also subjected to a thermal shift assay with purified Pru domain, and the binding was retained (Figure S16). This gave us confidence that incorporating the ester would aid in permeability, without compromising our binding to Rpn-13.

The toxicity of TCL-Ester was assessed in a variety of cell types, some with high reliance on proteasomal activity for survival (Ramos, Raji, MM.1R), and others with less reliance for survival (HEK and MRC-5) to demonstrate selectivity for hematological cancers, Figures 5A and B.[32,33] Since hematological cancers are sensitive to proteasome inhibition, toxicity in these cell lines could indicated that TCL-1 affects proteasome-mediated proteolysis through an interaction with Rpn-13. We dosed these five different cell types with increasing

concentrations of TCL-Ester. Excitingly, our results show that in the hematological cancer cell lines, the TCL-Ester derivative is toxic after 24 hrs with almost 100% cell death at 25–50 μM. In contrast, in the cell lines that rely less on proteasomal activity for survival we had hoped to see no significant cell death up to 50 μM. In both HEK-293T and MRC-5, TCL-Ester remains non-toxic even up to 50 μM with only ~50% death in HEK-293T cells at the highest concentration. These results are promising to show TCL-1 as a selective inhibitor of Rpn-13, as its toxicity profile is similar to that of the other known non-covalent Rpn-13 inhibitor KDT-11.[19]

From these studies, we have identified a non-covalent binder to Rpn-13, one of the ubiquitin receptors of the proteasome's 19S RP and quantified the binding affinity by NMR as K_D ~26 μM. Non-covalent binders to Rpn-13 are of significant interest for their potential application to be used as an inhibitor or even as a bifunctional degrader to either degrade Rpn-13 or potentially recruit proteins closer to the 26S proteasome for degradation.[34,35] This method of directing a protein to the 26S proteasome via a ubiquitin-independent mechanism for degradation has recently been validated.[36]

NMR chemical shift analysis supports binding of TCL-1 at a region of Rpn-13 near the Rpn2 association. Although these studies do not provide a mechanism for the toxicity observed with the hematological cancer cell lines, after preparing more potent analogs of TCL-1, we will more thoroughly study how they are eliciting their cytotoxic effect. The generation of the TCL-Ester analog highlights that the scaffold can be modified and still retain binding to Rpn-13's Pru domain, and we hope future medicinal chemistry optimization can produce a more potent and selective binder.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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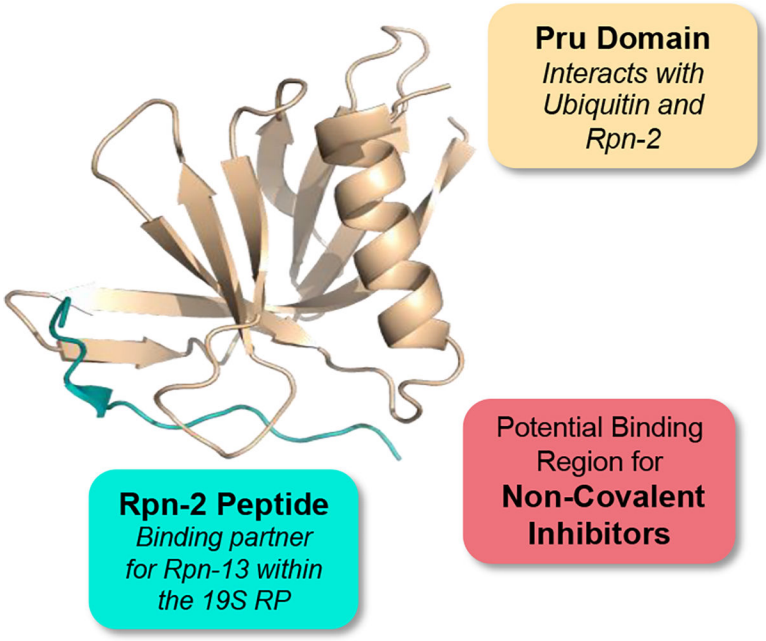

Figure 1.

Structure of the Pru domain (colored tan) of Rpn-13. Targeting the Pru domain has been shown to be a promising target in treatment of multiple myeloma. The interaction of Rpn-13 with its binding partner in the 19S RP complex, Rpn-2 (amino acids 940–953 shown in cyan), is essential for Rpn-13's ability to bind ubiquitinated proteins. Structure adapted from PDB File: 6CO4

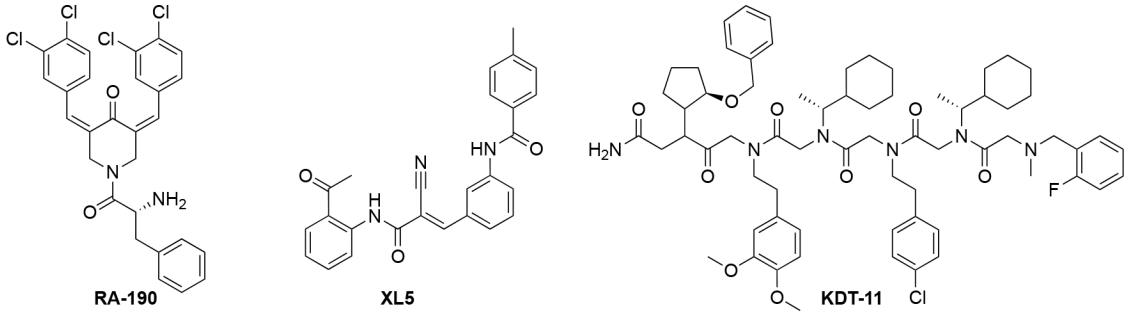

Figure 2.

Structures of covalent Rpn-13 inhibitors RA-190, XL-5 and the reversible peptoid binder KDT-11. Currently KDT-11 is the only reversible binder to the Pru doman. We are seeking to discover a reversible binder that is more drug like than KDT-11.

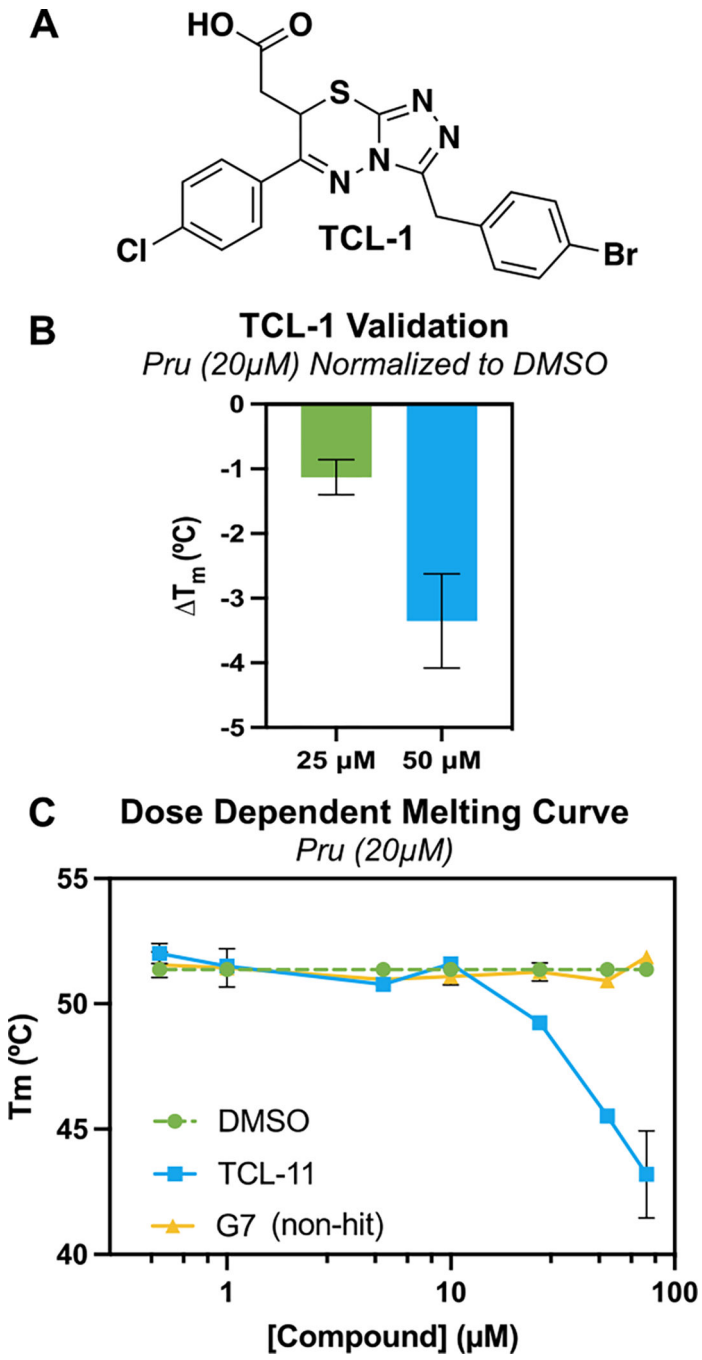

Figure 3.

A. Structure of validated compound TCL-1. **B.** Thermal shift results of TCL-1 at 25 μM and 50 μM with the Pru domain compared to DMSO ($\Delta T_m = 0$). **C.** Dose-dependent melting curve of TCL-1 and a negative control, G7.

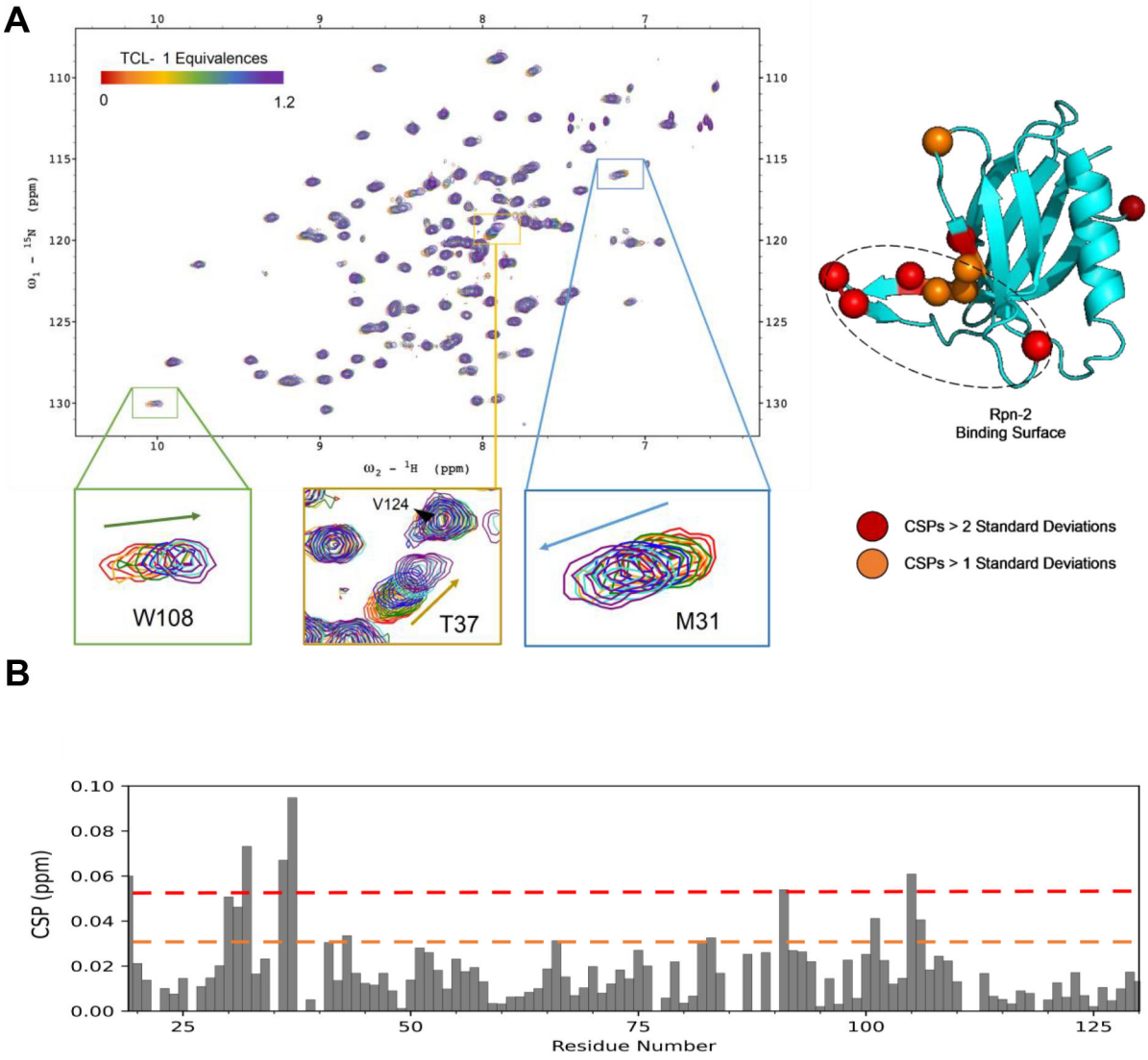

Figure 4.

A. HSQC spectral overlay of the 15N-labeled Pru domain (200 μM) titrated with TCL-1 at L/P 0, 0.2, 0.4, 0.6, 0.8 and 1. Selected amino acid shifts W108 (green), T37 (gold), and M31 (blue) show chemical shift perturbations CSP = $\sqrt{\left(0.154*\Delta^{15}\mathrm{N}\right)^2+\left(\Delta^{1}\mathrm{H}\right)^2}$ in the presence of TCL-1 mapped onto the structure with spheres. **B.** Chemical shift perturbations (CSPs) for each amino acid residue on the Pru domain after TCL-1 addition. Orange line = CSP > 1 standard deviation, Red line = CSP > 2 standard deviation.

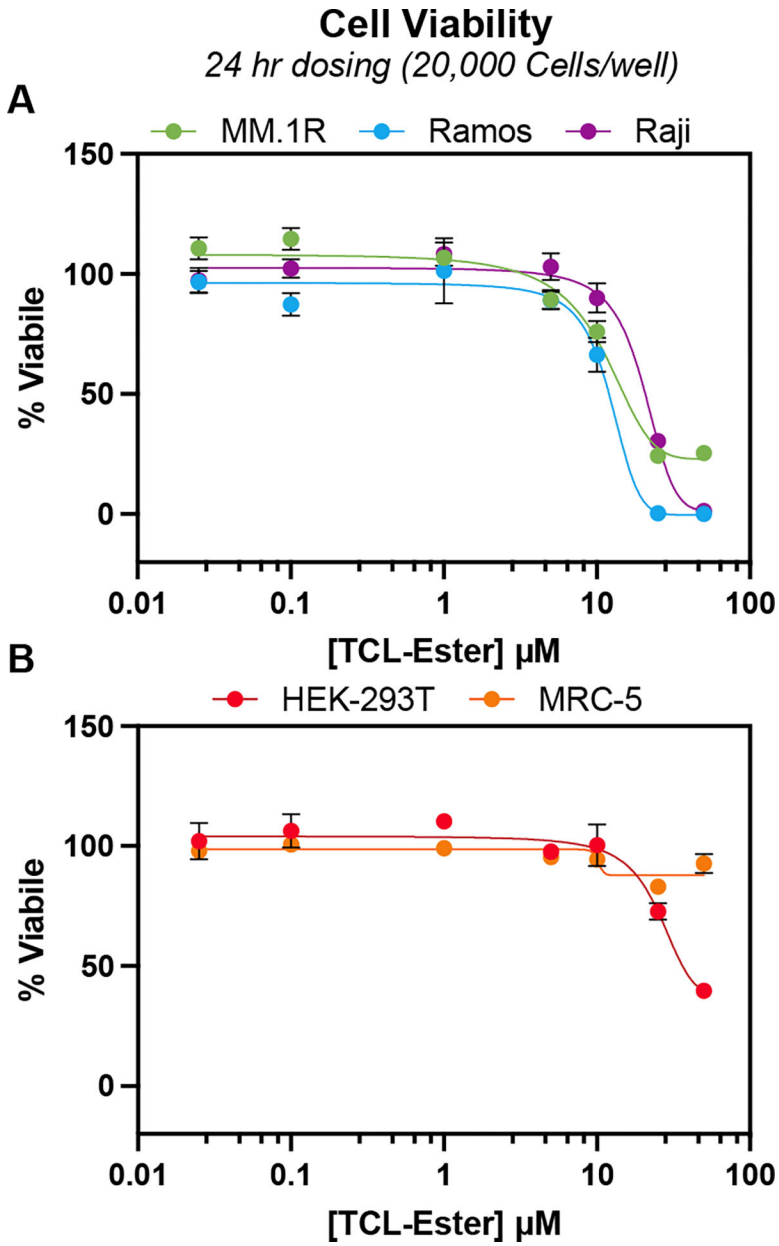

Figure 5.

Cell viability of TCL-Ester in **(A)** hematological cancer cell lines and **(B)** cell lines that rely less on high proteasomal activity for survival. Cells plated at 20,000 cells/well in a 96 well plate, then subjected to dosing with compound from 0 – 50 µM for 24 hr. MRC-5 (orange) and HEK-293T (red) show little toxicity after 24 hrs, while Raji (purple), Ramos (blue), and MM.1R (green) show more significant cell death using TCL-Ester.