Development of a Cyclic Peptide Inhibitor of the p6/UEV Protein-Protein Interaction.

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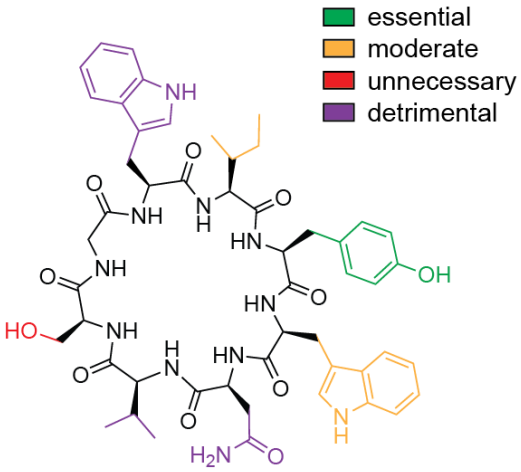
Supporting Information Placeholder

ABSTRACT: The budding of HIV from infected cells is driven by the protein-protein interaction between the p6 domain of the HIV Gag protein and the UEV domain of the human TSG101 protein. We report the development of a cyclic peptide inhibitor of the p6/UEV interaction, from a non-cell-permeable parent that was identified in a SICLOPPS screen. Amino acids critical for the activity of the parent cyclic peptide were uncovered using alanine-scanning, and a series of non-natural analogues synthesized and assessed. The most potent molecule disrupts the p6/UEV interaction with an IC_{50} of 6.17 ± 0.24 μM by binding to UEV with a K_d of 11.9 ± 2.8 μM. This compound is cell permeable and active in a cellular virus-like particle budding assay with an IC_{50} of ~2 μM. This work further demonstrates the relative simplicity with which the potency and activity of cyclic peptides identified from SICLOPPS libraries can be optimized.

The budding of immature virion from the membrane of an infected cell employs several host proteins and as such, appears ideal for therapeutic intervention by a protein-protein interaction inhibitor. In the case of HIV, the host's 'endosomal sorting complexes required for transport' (ESCRT) machinery is critical for budding.[1] In its normal function, the ESCRT regulates the entry of ubiquitin-tagged proteins into the endosome through vesicle formation.[2] In HIV infected cells, the viral HIV Gag polyprotein recruits the ESCRT through a protein-protein interaction (PPI) between a Pro-Thr-Ala-Pro (PTAP) motif from the p6 domain of HIV Gag and the UEV domain of human 'tumor suppressor gene 101' (TSG101) from ESCRT-1.[3, 4] The p6/UEV PPI is crucial to HIV budding as either depletion of TSG101 by siRNA, deletions of p6 from the HIV-Gag precursor, or mutations in the PTAP motif arrest the process.[5-7] While the heavy reliance on host proteins suggest budding to be an ideal step for therapeutic intervention, there are currently no therapeutic agents that target HIV budding.



CP11: *cyclo*-SGWIYWNV

Figure 1. Mapping of the alanine scanning data from Table 1 onto the structure of CP11.

The first-in-class inhibitor of the p6/UEV PPI is a cyclic octapeptide (*cyclo*-SGWIYWNV) named CP11.[8] This molecule was identified from a genetically encoded 'split-intein circular ligation of peptides and proteins' (SICLOPPS) library of 3.2 million members.[8] SICLOPPS enables the intracellular generation of cyclic peptide libraries, which has been combined with a variety of cell-based screens for the discovery of a wide range of inhibitors.[9-11] In this case, a SGWXXXXX library (X = any proteinogenic amino acid) was screened in a bacterial reverse two-hybrid system (RTHS)[12, 13] that linked the life/death of an engineered *E. coli* to the p6/UEV PPI.[8] CP11 was not active when directly added to cells, but when CP11 was conjugated to the cell-penetrating Tat peptide it was found to inhibit the production of virus-like particles (VLPs) from human cells with an IC_{50} ~7 μM.[8] We have also recently reported a bicyclic inhibitor of this interaction named XY3-3 (identified from a genetically encoded lanthipeptide library screened in the same p6/UEV RTHS) that binds UEV with a K_d of 4.0 ± 3.3 μM.[14] However, this compound also required conjugation

with a Tat-tag for activity in cell-based assays. Given the potential significance of a p6/UEV PPI inhibitor, both as a chemical tool and as the starting point for drug development, we set out to improve the activity and cell permeability of CP11.

Table 1. Assessing the effect of alanine-scanning analogues of CP11 (*cyclo*-SGWIYWNV) on disruption of the p6/UEV PPI by ELISA.

Name	Sequence	IC_{50}
CP11	*cyclo*-SGWIYWNV	48.6 ± 2.1 μM
A1	*cyclo*-AGWIYWNV	42.9 ± 11.6 μM
A2	*cyclo*-SAWIYWNV	54.9 ± 23.3 μM
A3	*cyclo*-SGAIYWNV	33.7 ± 4.3 μM
A4	*cyclo*-SGWAYWNV	79.3 ± 18.1 μM
A5	*cyclo*-SGWIAWNV	296.3 ± 24.3 μM
A6	*cyclo*-SGWIYANV	60.8 ± 31.4 μM
A7	*cyclo*-SGWIYWAV	25.8 ± 3.9 μM
A8	*cyclo*-SGWIYWNA	28.7 ± 11.0 μM

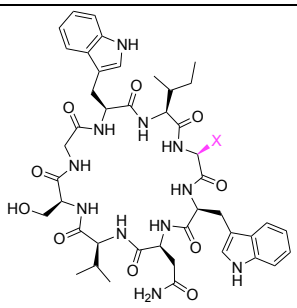
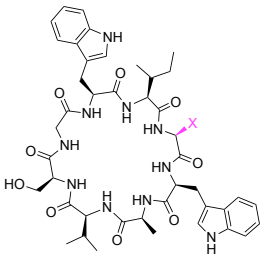
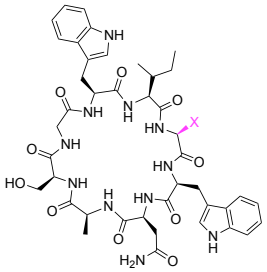
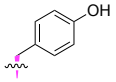
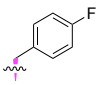
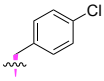
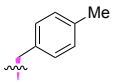
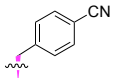
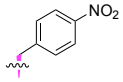
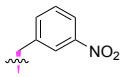
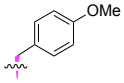
We began by identifying the amino acids in CP11 that are critical for its activity by alanine scanning. Eight derivatives of CP11 were synthesized, with one amino acid substituted for alanine in each molecule (Table 1 and Figure 1). We assessed and ranked these peptides for their ability to disrupt the p6/UEV PPI via an enzyme-linked immunosorption assay (ELISA). The parent CP11 was found to disrupt the p6/UEV PPI with an IC_{50} of 48.6 ± 2.1 μM by ELISA (Table 1 and Supplemental Figure 1). The SICLOPPS library used to encode CP11 contained an invariable SGW motif in every member of the library, chosen to aid peptide synthesis and identification by HPLC; replacing each amino acid in the SGW motif with alanine did not reduce the activity of CP11 (peptides A1-A3, Table 1), suggesting that this set motif does not engage with the target protein. We next assessed the effect of replacing each residue in the randomized region of CP11 with alanine. The largest loss of activity (6-fold) was observed when tyrosine was replaced with alanine; *cyclo*-SGWIAWNV disrupted the targeted PPI with an IC_{50} of 296.3 ± 24.3 μM (A5, Table 1). The isoleucine next to the tyrosine was identified as the next most important residue; this change only resulted in a 2-fold loss of activity (*cyclo*-SGWAYWNV, A4, IC_{50} of 79.3 ± 18.1 μM). Replacing the tryptophan (*cyclo*-SGWIYANV, A6) in the randomized region of CP11 with alanine resulted in minor loss of activity (IC_{50} 60.8 ± 31.4 μM); indicating that this residue has little interaction with the target.

In an effort to improve the potency of CP11, we synthesized several tyrosine analogues containing a handful of non-natural aromatic amino acids. Given that 2 of our alanine-scanning analogues (peptides A7 and A8, Table 1) were more potent than CP11, these scaffolds were also used as a separate starting point for incorporation of the non-natural amino acids (Table 2). These compounds were prepared by solid-phase peptide synthesis and purified. The activity of the resulting library of cyclic peptides was measured using the p6/UEV ELISA. The most potent derivative of CP11 (IC_{50} of 12.8 ± 1.1 μM, Table 2 and Supplementary Figures 2-4) contained a 4-methylphenylalanine in place of tyrosine, suggesting that hydrogen bonding from the tyrosine hydroxyl group did not contribute to the activity of CP11. The cyclohexylalanine derivative showed similar activity (IC_{50} of 13.6 ± 0.8 μM). Interestingly, most of the A7 (*cyclo*-SGWIYWAV) derivatives were more active than their CP11 counterparts, with the most potent being 4-chloro-phenylalnine (IC_{50} of 6.2 ± 0.2 μM). Interestingly, the majority of the A8 (*cyclo*-SGWIYWNA) series were less potent than their A7 counterparts ($IC_{50} \geq 14.5 \pm 1.0$ μM). Overall, the most potent derivative was *cyclo*-SGWI(4-Cl-F)WAV, named KRL74 **(**Figure 2a), which disrupted the p6/UEV PPI with an IC_{50} of 5.44 ± 1.87 μM (Figure 2b); a 7-fold improvement over the parent molecule CP11.

We next sought to identify the binding partner of KRL74 by assessing the affinity of KRL74 for p6 and UEV by microscale thermophoresis (MST). KRL74 was found to bind to UEV with a K_d of 11.9 ± 2.8 μM (Figure 2c), but no binding of KRL74 to p6 was observed (Supplemental Figure 5). For comparison, CP11 binds UEV with a K_d of 67.1 ± 7.1 μM (Supplementary Figure 6); These results indicate that KRL74 disrupts the p6/UEV PPI by binding to UEV and corroborate the previously reported indirect evidence for CP11 binding to UEV.[8]

We next compared the cell permeability of KRL74 to CP11 by dosing HeLa cells with a mixture containing 10 μM of both cyclic peptides. Cells were incubated overnight prior to washing to remove cyclic peptides form the medium, followed by lysis and pelleting of membrane and cell debris by centrifugation. HPLC analysis of the remaining cell lysate revealed the presence of KRL74, but not CP11 (Figure 2d). This data indicates that our optimized p6/UEV inhibitor KRL74 is cell permeable, and confirms our prior indirect observation that CP11 is not cell permeable. Furthermore, the data suggests that KRL74 may be used in cell-based assays without the need for conjugation to a permeability tag (e.g. a Tat-tag), as was the case for CP11. The observed difference in cell permeability between these molecules is interesting as the chemical changes between the two molecules are relatively minor.

Table 2. Assessing the activity of CP11 analogues containing non-natural tyrosine derivatives on the p6/UEV PPI by ELISA. Compounds that were more potent than CP11 are shown in green (darker shades representing higher potency) and those that were weaker than CP11 are shown in red (darker shades representing weaker activity). For individual plots see Supplementary Figures 2 to 4.

X =	 *cyclo*-SGWI**X**WNV	 *cyclo*-SGWI**X**WAV	 *cyclo*-SGWI**X**WNA
	48.6 ± 2.1 µM	25.8 ± 3.9 µM	28.7 ± 11.0 µM
	59.6 ± 5.4 µM	21.2 ± 0.4 µM	26.8 ± 1.9 µM
	18.9 ± 2.3 µM	31.7 ± 2.9 µM	15.9 ± 3.0 µM
	16.2 ± 5.1 µM	6.2 ± 0.2 µM	18.4 ± 1.8 µM
	12.8 ± 1.1 µM	8.2 ± 0.2 µM	14.5 ± 1.0 µM
	29.6 ± 3.8 µM	19.8 ± 1.4 µM	20.3 ± 1.2 µM
	33.8 ± 2.9 µM	29.5 ± 3.0 µM	48.2 ± 4.0 µM
	56.1 ± 8.4 µM	17.8 ± 2.9 µM	27.3 ± 4.3 µM
	38.9 ± 7.7 µM	11.8 ± 1.0 µM	63.4 ± 2.6 µM
	33.2 ± 2.4 µM	50.1 ± 3.5 µM	44.1 ± 12.0 µM
	13.6 ± 0.8 µM	6.9 ± 0.6 µM	26.9 ± 2.4 µM
	15.9 ± 5.4 µM	37.8 ± 11.4 µM	no activity

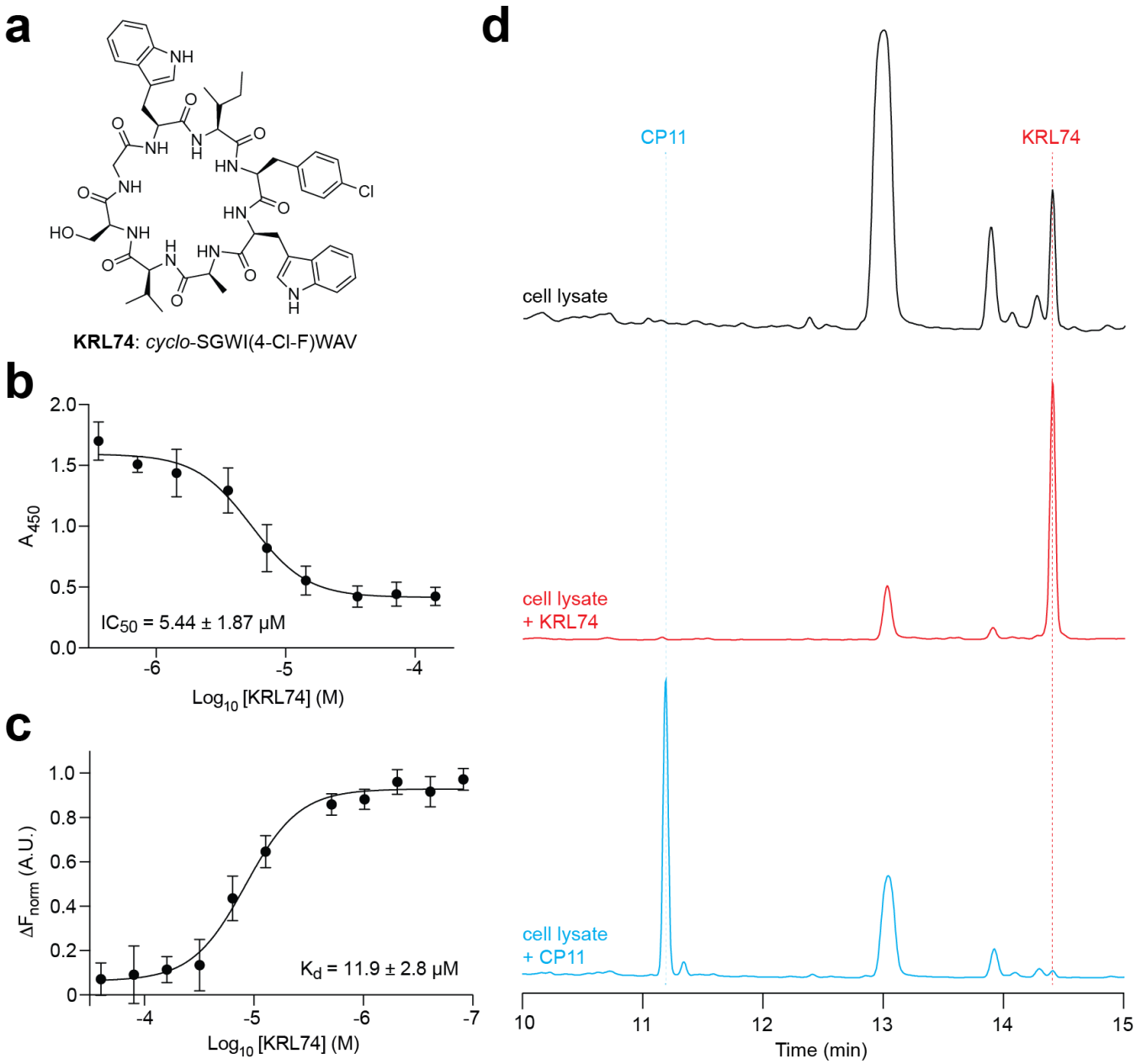


Figure 2. Characterizing the activity of KRL74 *in vitro* and in cells. a) chemical structure of KRL74. b) KRL74 disrupts the p6/UEV PPI with an IC_{50} of 5.44 ± 1.87 µM by ELISA. c) KRL74 binds to UEV with a K_d of 11.9 ± 2.8 µM by MST. d) HPLC trace (A280) of lysate from HeLa cells dosed with KRL74 and CP11 (10 µM each) shows the presence of KRL74 only (black line). HPLC trace (A280) of cell lysate spiked (post lysis) with synthetic KRL74 (red line) or CP11 (blue line) used as controls. For all data, error bars represent SEM (n=3).

We assessed the activity of KRL74 in a previously reported cell-based virus-like particle (VLP) budding assay.[8] The assay uses HEK293T cells transfected with a plasmid encoding Gag-GFP protein. The HIV-1 Gag-GFP fusion protein forms VLPs that bud from these cells in a process dependent on the p6/UEV PPI.[6, 15] Thus an inhibitor of the p6/UEV PPI will be expected to reduce the release of VLPs in this assay. Transfected cells were dosed with KRL74, DMSO (0.2%) or water (control) and the VLPs produced by these cells collected and quantified by western blot to GFP. We observed a dose-dependent reduction in VLPs isolated from the supernatant of cells treated with KRL74 (Figure 3a), with an IC50 of ~2 µM. This represents a 2-fold improvement on the cell-based activity of Tat-tagged CP11, but without the need for a permeability tag on KRL74.

As loss of TSG101 has previously been shown to be lethal in cells and in vivo,[16, 17] we assessed the toxicity of KRL74 to HEK293T cells via a commercial luminescence-based toxicity assay. We observed no toxicity from this compound at concentrations up to twice that used in the budding assay (Supplementary Figure 7).

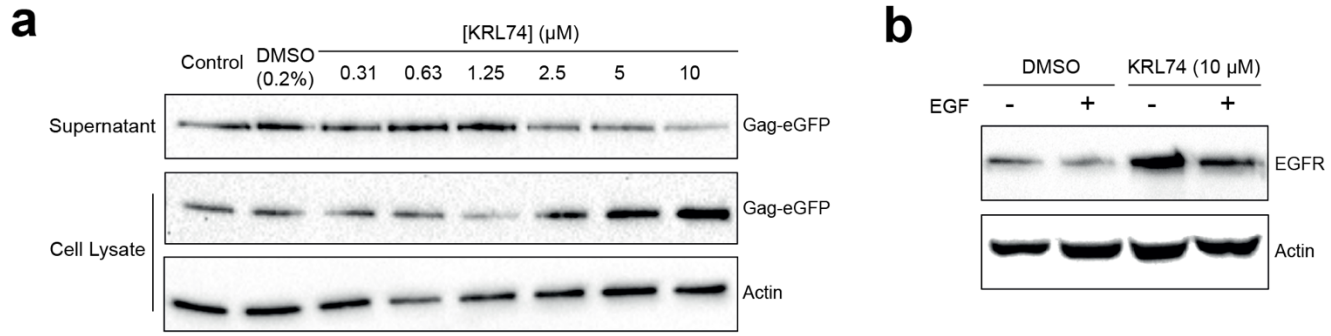


Figure 3. Characterizing the effect of KRL74 in cells. a) Western Blot of VLP assay where HEK293T cells were transfected with Gag-eGFP and then treated with peptide. b) Western Blot of EGFR assay showing the effect of peptide on downregulation of EGFR in HeLa cells.

The cell-based activity of KRL74 was further assessed via the UEV-mediated trafficking of the endosomal growth factor receptor (EGFR). EGFR is activated by endosomal growth factor (EGF) and subsequently targeted for degradation by the ESCRT machinery.[2, 18-20] This process relies on the PPI of UEV with a protein called HRS via the same hotspot region as the p6/UEV PPI (but via a PSAP rather than PTAP motif).[21] As previously observed for CP11 and XY3-3,[8, 14] KRL74 would be expected to also inhibit the ESCRT-mediated degradation of EGFR by binding to UEV and preventing its interaction with HRS. HeLa cells expressing endogenous EGFR were treated with KRL74 (10 μM), or DMSO (0.2%), and after overnight incubation were either treated with EGF for 90 minutes or left untreated. The effect of EGF and KRL74 on EGFR levels was assessed by western blot. We observed a reduction in EGFR levels when cells were treated with EGF (Figure 3b, lane 1 v lane 2). As expected, treatment with KRL74 resulted in increased EGFR levels (Figure 3b, lane 3 v lane 1), and cells treated with both KRL74 and EGF also showed higher levels of EGFR than the corresponding cells treated with EGF alone (Figure 3b, lane 4 v lane 2). Together, the above data demonstrates that our p6/UEV PPI inhibitor (KRL74) is active in cells.

In summary, we have reported the derivatization of a cyclic peptide p6/UEV PPI inhibitor into a more potent and cell permeable cyclic peptide. This molecule may be used as a tool for studying the ESCRT machinery and/or of HIV budding in cells. KRL74 may also be further developed towards the clinic. As the budding of Ebola virus also relies on usurping the ESRCT machinery via the binding of a PTAP motif to UEV,[22] KRL74 may have potential beyond inhibiting HIV budding. While the cyclic peptide reported here is slightly less potent than the previously reported bicyclic lanthipeptide p6/UEV PPI inhibitor XY3-3,[14] it is active in cells without the need for a permeability tag, and is significantly simpler to prepare by chemical synthesis. Both of these advantages increase its value as a chemical tool. On a broader level, this work adds to increasing evidence of the relative ease with which the activity of SICLOPPS-derived cyclic peptides may be optimized into more potent, cell permeable molecules by incorporating non-natural amino acids post-selection.[9, 11, 23-25]

ASSOCIATED CONTENT

Supporting Information.
The Supporting Information is available free of charge on the ACS Publications website.
Supporting figures and material and methods (PDF)

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