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Expression and purification of active Receptor Interacting Protein 1 kinase using a baculovirus system

Jenny L. Maki^a, J. Tres Brazell^a, Xin Teng^b, Gregory D. Cuny^{b,1}, and Alexei Degterev^{a,*}

^aDepartment of Biochemistry, School of Medicine, Tufts University, Boston, MA 02111

^bLaboratory for Drug Discovery in Neurodegeneration, Harvard NeuroDiscovery Center, Brigham & Women's Hospital and Harvard Medical School, Cambridge, MA 02139

Abstract

Receptor Interacting Protein 1 (RIP1) kinase is one of the key mediators of tumor necrosis factor alpha (TNF- α) signaling and is critical for activation of necroptotic cell death. We developed a method for expression of recombinant kinase, utilizing baculovirus co-infection of Cdc37, an Hsp90 co-chaperone, and RIP1-His, followed by a two-step purification scheme. After optimization, 1-3 mg of highly purified RIP1 kinase was typically obtained from a 1 L of Sf9 cells. The recombinant protein displayed kinase activity that was blocked by RIP1 inhibitors, necrostatins. The purified protein was used to develop a simple and robust thermal shift assay for further assessment of RIP1 inhibitors.

Keywords

RIP1 kinase; Cdc37; necrostatins; baculovirus; thermal shift assay

Introduction

Cellular necrosis plays a critical role in many physiological and pathological processes but has traditionally been viewed as unregulated cell death [1]. In the past several years, a new form of regulated necrosis termed necroptosis has emerged [2,3,4,5,6]. This form of necrosis has been implicated in the responses to a variety of different signals including tumor necrosis factor-alpha (TNF- α , tumor necrosis factor ligand superfamily member 6 (FasL), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [1] as well as in developmental cell death in apoptosis-deficient animals [7] and in a variety of pathologic injuries [8]. The regulation of necroptosis revolves around a unique signaling pathway involving two homologous kinases, receptor interacting protein 1 and 3 (RIP1 and RIP3, respectively), which form a cytosolic signaling complex during necroptosis [4].

RIP1 is a serine/threonine protein kinase, which consists of an N-terminal kinase domain, an intermediate domain, and a C-terminal death domain [9]. Through its death domain, RIP1

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*Corresponding author at: Department of Biochemistry, School of Medicine, Tufts University, Stearns 703, 136 Harrison Ave, Boston, MA 02111, USA. Phone: +1 617 636 0491, Fax: +1 617 636 2409 alexei.degterev@tufts.edu.

¹Current Address: Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston, 549A Science and Research Bldg 2, Houston, TX 77204

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interacts with the TNF- α receptor complex and the state of RIP1 determines if the cell will activate the nuclear factor κ B (NF κ B) pathway or die through apoptosis or necroptosis [10]. During NF κ B activation, RIP1 remains associated with the membrane bound TNF receptor (TNFR) complex (complex I) and is K63-linked polyubiquitinated on K377. This ubiquitination prevents RIP1 dissociation from complex I [11]. In TNF- α induced cell death, RIP1 dissociates from complex I to form complex II, which directs cells either to apoptotic or necroptotic death. Complex II consists of RIP1, TNF- α receptor-associated death domain (TRADD), Fas-associated death domain (FADD) and caspase-8 and is localized to the cytoplasm [3]. RIP1 kinase activity can specifically contribute to the activation of caspase-8 in the absence of cellular inhibitor of apoptosis proteins (cIAPs) [12,13]. When caspase-8 is inhibited, RIP1 kinase activity induces the formation of the RIP1/RIP3 necrosome and the initiation of necroptosis [4].

Since necrosis plays a critical role in pathological injuries and diseases, a cell-based screen was designed to find inhibitors of TNF- α induced necroptosis [2]. Several structurally dissimilar small molecules were discovered and termed necrostatins (Necs). Interestingly, three of these molecules (Nec-1, Nec-3, and Nec-4) specifically inhibit the kinase activity of RIP1 but have no effect on RIP3 kinase activity [14]. To further understand the activity and inhibition of RIP1 kinase, we optimized the large-scale expression and purification of an active kinase domain of RIP1 protein, aa 8-322 with a C-terminal His tag, using co-baculovirus infection with the Hsp90 co-chaperone, Cdc37 [15]. This co-infection method is amendable to destabilizing mutants or other kinases that are prone to aggregation and are clients of Hsp90. The recombinant RIP1 kinase activity was confirmed using a radiometric autophosphorylation assay and was inhibited by necrostatins. This protein was further used to develop a thermal shift analysis assay, which can be used for screening novel RIP1 inhibitors.

Material and Methods

Materials

All chemicals and reagents were obtained from Sigma, Fisher, or VWR unless otherwise indicated.

Construction of expression vector of RIP1 8-322-His

The kinase domain of human RIP1 (aa 8-322) was amplified using Phusion High-Fidelity Polymerase (New England Biolabs) with the primers 5'-GCGTACCGAGATCTATGAATGTCATTAAGATGAAATCC-3' and 5'-GAGGAATTCC TAGTGGT GATGGTGGT GATGTTGAAGAGACTGCATTCTC-3' incorporating a C-terminal 6-His tag. The resulting PCR product was digested with BglII and EcoRI restriction enzymes (New England Biolabs) and ligated into the same restriction sites in the pVL1392 vector (BD Biosciences) creating the pVL1392-RIP1 8-322-His plasmid. The correct sequence of the plasmid was verified by DNA sequencing (Core Facility, Tufts University).

Sf9 cell culture and baculovirus generation

Spodoptera frugiperda (Sf9) insect cells were grown in T175 flasks at 27 °C in Sf-900 II SFM medium (Gibco/Invitrogen). The RIP1 8-322-His baculovirus (termed RIP1-His) was generated using the BD BaculoGold Transfection Buffer A & B Set (BD Biosciences) according to the manufacturer's protocol by transfecting 2 μ g of pVL1392-RIP1 8-322-His plasmid and 0.5 μ g of linearized BaculoGold Bright DNA (BD Biosciences) into 2×10^6 Sf9 cells. The RIP1-His baculovirus was amplified to passage five (P5). Cdc37 baculovirus (FoldHelper-37, AB Vector, LLC) was also amplified in Sf9 cells.

Baculovirus titer determination

The titer of the Cdc37 baculovirus was determined using FastPlax Titer Kit (Novagen) following the manufacturer's protocol resulting in a titer of 3.2×10^6 pfu/ml. The titer of the RIP1-His baculovirus was determined by end-point dilution and GFP fluorescence detection. The 50% tissue culture infectious dose (TCID₅₀) was used to calculate the virus titer and converted to infectivity in plaque forming units (pfu/ml) [22]. The titer was calculated to be 1.7×10^6 pfu/ml.

Testing RIP1 8-322-His expression

Sf9 cells were plated in four 15-cm dish and infected with 100 μ l of 1.7×10^6 pfu/ml RIP1-His baculovirus, 10 μ l of Cdc37 baculovirus, 100 μ l of RIP1-His and 10 μ l of 3.2×10^6 pfu/ml Cdc37 baculoviruses or no baculovirus. The cells were grown at 27 °C for approximately 72 hours. Cells were resuspended in lysis buffer (40 mM HEPES pH 7.3, 150 mM NaCl, 0.5 mM NaF, 0.2 mM NaVO₃, 10 mM sodium pyrophosphate, 17.5 mM β -glycerolphosphate, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, and 50 μ g/ml PMSF) and incubated on ice for 15 minutes. After centrifugation, the supernatant was added to 20 μ l of HIS-Select Nickel Affinity Gel (Sigma) pre-washed with lysis buffer and incubated at 4 °C rotating for one hour. The Nickel Affinity Gel was washed three times with lysis buffer followed by three washes with nickel wash buffer (50 mM Tris, pH 8.0, 300 mM NaCl). The RIP1 8-322-His protein was eluted using nickel elution buffer (50 mM Tris, pH 8.0, 300 mM NaCl, 500 mM Imidazole). The eluted protein was tested in a radiometric kinase assay to determine activity.

Necrostatin Synthesis

Necrostatins were synthesized as previously described, Nec-1 [16], Nec-3 [17], Nec-4 [18].

Radiometric Gel Kinase Assay

The kinase reaction was performed as previously described [14,19].

Optimization of expression of RIP1-His

In a 6-well plate, 1×10^6 Sf9 cells were plated in each well. Each well was infected with 20 μ l of 1.7×10^6 pfu/ml RIP1-His baculovirus (multiplicity of infection (MOI) of 0.03) and 0, 0.5, 1, 2, 3, or 4 μ l of 3.2×10^6 pfu/ml Cdc37 baculovirus. The plate was incubated at 27 °C for approximately 72 hours and then checked for GFP expression. The cells were collected and lysed using 1X sample buffer. Samples were analyzed by western blot to check for optimal RIP1 expression.

In two 6-well plates, 1×10^6 Sf9 cells were plated in 10 wells. Each plated well was infected with RIP1-His baculovirus at MOI of 0.03 and Cdc37 baculovirus at MOI of 0.006 and incubated at 27 °C. After 12 hours, the cells were checked for GFP expression and one well of cells were collected and lysed using 1X sample buffer. The same protocol was used every 12 hours for the next three days. The cells were collected and lysed using 1X sample buffer. Samples were analyzed by western blot to check for optimal RIP1 expression.

Western blot analysis

Cell extracts were separated by 12% SDS-PAGE and transferred to Immobilon-FL membrane (Millipore) and probed anti-Tubulin (Cell Signaling) followed by anti-mouse IgG-HRP (Cell Signaling). Blots were detected using Luminata Classico Western HRP substrate reagent (Millipore) and x-ray film. Tubulin was used to normalize the amount of sample loaded in each lane. Normalized samples were probed with anti-RIP1 (Cell Signaling) followed by anti-rabbit IgG-HRP (Cell Signaling) secondary antibody.

Large-scale expression and purification of RIP1-His

One liter of Sf9 insect cells at a density of 3×10^6 cells/ml, grown in ESF921 Protein Free medium (Expression Systems), were infected with 3 ml of 1.7×10^6 pfu/ml RIP1-His baculovirus and 300 μ l of 3.2×10^6 pfu/ml Cdc37 baculovirus and incubated at 27 °C, shaking at 150 rpm/min. Approximately 60 hours post-infection, cells were harvested, resuspended in lysis buffer, and incubated on ice for 15 minutes. The cells were sonicated followed by centrifugation. The resulting supernatant was loaded onto a 3 ml HIS-Select Nickel Affinity Gel (Sigma) column. A step gradient program was used to wash and elute the RIP1-His protein: (1) 100% nickel wash buffer for 20 column volumes (CVs), (2) 98% nickel wash buffer and 2% nickel elution buffer (10 mM imidazole) for 10 CVs, (3) 90% nickel wash buffer and 10% nickel elution buffer (50 mM imidazole) for 10 CVs, (4) 100% nickel elution buffer (500 mM imidazole) for 10 CVs. Fractions containing RIP1-His from step 3 were combined and concentrated to about 1 ml. The protein was injected onto a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with SEC buffer (50 mM Tris pH 8.0, 150 mM NaCl, and 2 mM β -mercaptoethanol). RIP1-His was eluted in 1.5 CVs using an isocratic gradient of SEC buffer. The pure RIP1-His fractions were pool and concentrated. Glycerol (20%) and PMSF (1 mM) were added to the protein, which was aliquoted, flash frozen, and stored at -80 °C. A NanoDrop 2000 Spectrophotometer (Thermo Scientific) was used to determine the final concentration of the protein. The activity of each batch of protein was checked using the radiometric gel kinase assay.

Fluorescence Thermal Shift Analysis

The thermal shift of RIP1-His was measured in clear Light Cycler 480 multiwell plate 96 (Roche) with a final volume of 20 μ l. All components were diluted in thermal shift buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$) with final concentrations of 9 μ M RIP1-His, 180 μ M necrostatins, 5X Sypro Orange (Life Technologies), and 3% DMSO. In duplicate wells, RIP1-His and the compounds were added to the microplate and incubated at room temperature for 5 minutes. Compounds without RIP1-His were also run to determine if compounds interfered with fluorescence readout. Sypro Orange dye was added and the plate was measured using a LightCycler 480 instrument (Roche) with excitation at 465 nm and emission at 580 nm. The protein denaturation fluorescence data was collected using melting curves analysis mode from 25 to 85 °C in continuous acquisition mode with 10 acquisitions per °C and a ramp rate of 0.06 °C/s. The data was analyzed to calculate T_m using nonlinear regression with Boltzmann sigmoidal equation in GraphPad 5 using approximately two degrees of pre- and post-transition baseline data.

Results and Discussion

Expression of Active RIP1-His

In order to further understand the activity and inhibition of RIP1 kinase in vitro, we needed to develop an expression method to generate stable and catalytically active RIP1 kinase domain protein. The activation loop of RIP1 is similar to another serine/threonine kinase B-Raf [14], therefore, we made a construct of similar size to a crystallized B-Raf construct [15]. The RIP1 construct we designed encodes amino acids 8-322 with a C-terminal 6-His tag and was termed RIP1-His. Using small-scale Sf9 cell infections, we confirmed expression of recombinant RIP1-His protein (Figure 1A Lane 1 and 3). This protein displayed kinase activity in a ^{32}P autophosphorylation assay but lacked inhibition with the optimized RIP1 inhibitor Necrostatin-1 (Opt Nec-1, Figure 1B, C). Opt Nec-1 inhibits the activity of the baculovirus expressed GST-RIP1 fusion protein proteins [14] [19] suggesting that the His tagged version of RIP1 may not be folded correctly. RIP1 is a known client protein of Hsp90 in mammalian cells [20], therefore, we tested a co-infection of the Hsp90 co-chaperone Cdc37 and RIP1-His baculoviruses together in Sf9 cells. This strategy has

been used successfully for the expression of another Hsp90-dependent kinase, B-Raf [21]. RIP1-His purified from the mixed baculovirus infection was expressed and retained kinase activity and was inhibited by Opt Nec-1 (Figure 1A, C). This suggests that the RIP1-His is prone to misfolding and the addition of the Cdc37 chaperone aids in correct folding allowing for proper kinase activity and inhibition.

To further optimize the expression of RIP1-His, a titration of Cdc37 was performed. The RIP1-His baculovirus was generated using BaculoGold Bright DNA (BD Biosciences), which has green fluorescent protein (GFP) incorporated into the baculovirus genome. This feature allows monitoring of baculoviral infection by fluorescence microscopy [22]. The Cdc37 baculovirus does not express GFP enabling us to titer the appropriate amount of Cdc37 virus, which would not overwhelm the expression of RIP1-His. At higher amounts of Cdc37 baculovirus, the number of GFP positive cells started to decrease meaning that the Cdc37 infection was overtaking the RIP1-His infection (data not shown). Western blot analysis showed an increase in RIP1-His expression with increasing amounts of Cdc37 (Figure 2A). Thus, multiplicity of infections (MOI) of 0.034 for RIP1-His and 0.0064 for Cdc37 were selected for expressing RIP1-His. To further optimize the purification process, we conducted an infection time course from 12 to 84 hours of the combined Cdc37 and RIP1-His baculoviruses (Figure 2B). The optimal time for harvesting cells was approximately 60 hours post infection. Shorter time points resulted in fewer GFP positive cells (data not shown), while longer time points showed proteolysis and modification of the RIP1-His protein. Therefore, a low MOI of RIP1-His co-infected with Cdc37 for 60 hours is optimal for producing recombinant RIP1-His kinase.

Large scale expression and purification of recombinant RIP1-His

After the optimal small-scale expression conditions were determined, the infection conditions were expanded to 1 L shaking cultures of Sf9 cells. After growth at 27 °C in a shaking incubator for 60 hours post-infection, the cell lysate was loaded onto a His-select gel column. The protein was purified using step purification with the imidazole method outlined in materials and methods section. At 50 mM imidazole RIP1-His elutes from the column (Figure 3A, fractions 9-18) but there is also another fraction of the protein that eluted with 500 mM imidazole (Figure 3A, fractions 20 - 24). This high imidazole fraction is likely unfolded because it behaves similar to RIP1-His expressed without Cdc37 (data not shown). After His-select gel column purification, RIP1-His protein still contained some higher molecular weight contaminants so we employed a second purification step of size exclusion chromatography (SEC) (Figure 3B). The SDS-PAGE of the different fractions demonstrates that a small portion of the RIP-His comes out early and is associated with larger molecular weight proteins that are most likely chaperones (Figure 3C). This suggests that a small portion of RIP1-His is incorrectly folded but can be separated by SEC. The calculated molecular mass of RIP1-His by SEC is approximately 32 kD, which is slightly smaller than the predicated molecular weight of 37 kD. This suggests that RIP1-His is monomeric and has a structure that is not molten globular. We further confirmed that the major peak of RIP1-His eluting in fractions 14-17 contains catalytically active and Nec-1-inhibitable kinase using a ³²P autophosphorylation assay (Figure 3D).

In a final optimization step, we varied the Sf9 cell density at the time of infection with the combination of Cdc37 and RIP1-His baculoviruses infection to determine the optimal cell density range for infection. Although the yield of RIP1-His varies between preparations, 3.0×10^6 cells/ml on average yielded about 1-3 mg of highly purified RIP1-His protein (Table 1). Higher cell densities yielded more inactive protein and were not further investigated. Therefore, the combination of Cdc37 and RIP1-His co-infection and a two-step purification process results in active RIP1-His protein that could be inhibited with necrostatins. This co-

infection method using Cdc37 helps with the expression of mutant RIP1 kinase as well as other kinases that are aggregation-prone (data not shown).

Binding and Kinase Inhibition of RIP1-His with Necrostatins

To check if purified RIP1-His behaves the same as GST-RIP1 [19] and mammalian expressed RIP1 [14], we performed a radiometric kinase assay with different necrostatins as well as the corresponding inactive analogs (Figure 1A, 3E). The necrostatins are in three different structural classes: hydantoin-containing indole derivative (Nec-1), tricyclic derivative (Nec-3), and pyrrole derivative (Nec-4). As previously shown with other RIP1 proteins, RIP1-His is inhibited by the original Nec-1, optimized Nec-1, Nec-3, and Nec-4, but not by the corresponding inactive Nec-1 and Nec-3 analogs. To further test these inhibitors with RIP1-His, we developed a thermal shift assay (TSA) using Sypro Orange. Sypro Orange is a fluorescent dye that has low fluorescence in aqueous solution but increases in fluorescence in nonpolar environments such as binding to exposed hydrophobic residues [23]. Therefore, this fluorescent dye can be used to measure the thermal unfolding of proteins using a real time PCR machine. The binding of ligands or inhibitors to protein will shift the thermal melt (T_m) of a protein to a higher temperature if it stabilizes or lower temperature if it destabilizes [24]. To test necrostatin binding in TSA, RIP1-His was subjected to thermal denaturation in the presence of DMSO or necrostatins and Sypro Orange (Figure 4A). RIP1-His with DMSO has a T_m of 45.0°C. Correlating with the radiometric assay, the necrostatins shifted the T_m of RIP1-His to a higher temperature indicating that they stabilized the structure (Figure 4B, Table 2). In general in TSA, a true binding event occurs if there is a shift in T_m of 2 °C or higher [24]. This is in agreement with our results where the inactive Nec-1 and Nec-3 analogs shift the T_m of RIP1-His less than 2 °C, while the necrostatins shifted the T_m by more than 5 °C. Unfortunately, Nec-4 and Sypro Orange interact with each other preventing TSA analysis with this inhibitor. TSA with RIP1-His is a highly reproducible assay that can be used as a primary screen for novel inhibitors of RIP1 kinase.

Conclusions

In this study we developed a co-baculovirus (Cdc37 and RIP1-His) infection method to express active RIP1 kinase domain protein. The use of the BaculoGold baculovirus with RIP1-His enabled us to modulate the amount of the molecule chaperone Cdc37 used during the infection to yield the greatest amount of active RIP1-His protein. Using a two-column purification scheme, we purify approximately 1-3 mg of RIP1-His protein from 1 L of infected Sf9 cells. This dual, low MOI infection method is amendable to RIP1 mutants as well as other kinases prone to aggregation. Recombinant RIP1-His was inhibited by necrostatins and was used in the development of a simple and robust thermal shift assay.

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Abbreviations

RIP1 receptor interacting protein 1 kinase

RIP3	receptor interacting protein 3 kinase
TNF-α	tumor necrosis factor alpha
NFκB	nuclear factor κ B
Necs	necrostatins
TCID₅₀	tissue culture infectious dose
MOI	multiplicity of infection
CV	column volume
EV	elution volume
TSA	thermal shift assay

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Highlights

- RIP1 kinase expressed with Cdc37 co-chaperone results in the expression of active kinase that is inhibited by necrostatins.
- Size-exclusion chromatography separates active and inactive proteins indicating that active RIP1-His protein is monomeric.
- Purified RIP1-His was used to develop a thermal shift assay with Sypro Orange.

Figure 1.

Expression of active RIP1-His. A. Coomassie blue stained 12% SDS-PAGE gel of different baculovirus infected samples purified using His-select beads and eluted with 500 mM imidazole: M – molecular weight marker, 1 – cells alone, 2 – Cdc37 infected cells, 3 – RIP1-His infected cells, 4 – Cdc37 and RIP1-His infected cells. B. Structures of the different necrostatins used in this study. C. ³²P autophosphorylation kinase reactions from the eluents of RIP1-His infection (no Cdc37) and Cdc37 and RIP1-His co-infection, treated with either DMSO (D) control or 30 μM of optimized RIP1 inhibitor Nec-1 (N1). RIP1-His expressed alone was not inhibited by Opt Nec-1.

Figure 2.

Optimization of RIP1-His co-baculovirus infection with Cdc37. A. The expression of RIP1-His was tested by mixing RIP1-His baculovirus with increasing concentrations of Cdc37 baculovirus. The expression of RIP1-His was detected by western blot using a RIP1 antibody. The lanes are labeled according to the MOI of Cdc37. Tubulin was used to normalize the amount of sample in each lane. B. Time-course of infection was performed by taking samples every 12 hours after co-baculovirus infection using MOIs of 0.034 for RIP1-His and 0.0064 for Cdc37. The expression of RIP1-His was monitored by western blot. At 72 hours post-infection, RIP1-His starts to get modified and degraded so 60 hours was determined to be optimal.

Figure 3.

Purification and activity of RIP1-His. A. RIP1-His and Cdc37 baculoviruses were coinfecting into 1L of Sf9 cells at a density of 3×10^6 cells/ml. Sixty hours post-infections, the cells were lysed as described in the materials and methods. The supernatant was loaded onto a 3 ml His-select column. Coomassie blue stained 12% SDS-PAGE gels represent the different imidazole steps from the His-select column: M – molecular weight marker, lanes 1-2 flow through from loading, lanes 3-5 0 mM imidazole step, lanes 6-8 10 mM imidazole step, lanes 9-18 50 mM imidazole step and lanes 19-28 500 mM imidazole step. B. Chromatogram of the absorbance at 280 nm of the 50 mM imidazole eluted fractions from A injected onto a Superdex 200 SEC column (GE Healthcare). C. Coomassie blue stained 12% SDS-PAGE gel of Superdex 200 SEC elution volume collected in 1 ml fractions. D. Elution volume fractions from B were tested in the radiometric kinase assay with protein concentration of 1 μ M with either DMSO (D) or 15 μ M opt Nec-1 (N1). E. The final purified RIP1-His protein at 2 μ M was tested in the radiometric kinase assay with the various necrostatins at 30 μ M (Figure 1B). RIP1-His is inhibited by necrostatins but not by the inactive analogs.

Figure 4.

Thermal shift assay with RIP1-His A. RIP1-His at 9 μ M in the presence of 3% DMSO or 18 μ M inhibitors was subjected to thermal denaturation using Sypro Orange as a fluorescent tracer. Each sample was run in duplicate and one representative thermal trace is shown. B. The T_m for RIP1-His with and without inhibitors was calculated as described in the materials and methods. The shift in T_m from RIP1-His DMSO to RIP1-His inhibitors demonstrates that ori Nec-1, opt Nec-1, and Nec-3 increased RIP1-His T_m , while Nec-1i and Nec-3i have a minimal effect on RIP1-His T_m demonstrating that they do not bind.

Table 1

Sf9 cell density effect on RIP1-His protein yield

Cell Density (cells/ml)	RIP1-His/1L culture (mg)	Number of Preps
2.0×10^6	1.6 ± 0.1	5
2.2×10^6	2.0 ± 1.1	3
3.0×10^6	2.6 ± 1.2	8
3.5×10^6	2.3 ± 0.9	7
5×10^6	4	1

Table 2

Thermal shift values

	T_m (°C)	ΔT_m (°C)
DMSO	45.0 ± 0.3	0.0
Ori Nec-1	50.4 ± 0	5.4
Ori Nec-1i	45.3 ± 0.07	0.3
Opt Nec-1	51.9 ± 0.01	6.9
Nec-3	52.2 ± 0.02	7.2
Nec-3i	46.5 ± 0.01	1.5

n=2