Dissecting RIPK3 Autophosphorylation with the Potent Necroptosis Inhibitor UH15-38

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Background

Programmed cell death is a fundamental component of the cell cycle, occurring during normal development or in response to stress conditions such as tissue injury or disease (Raff, 1998). Apoptosis is the most extensively studied and historically regarded as the dominant form of programmed cell death; however, alternative pathways also exist. This study focuses on necroptosis, a distinct form of regulated necrosis. In contrast to apoptosis, which is generally immunologically silent and supports tissue homeostasis, necroptosis is activated downstream of diverse inflammatory and innate immune receptors and has been implicated in a wide spectrum of human diseases, including neurodegenerative, cardiovascular, infectious, and inflammatory disorders, as well as cancer (Yuan, 2005). Although emerging evidence suggests that necroptosis may contribute to certain beneficial immunosignaling processes (Ye, 2023), it remains a therapeutic target for inhibition because it is not necessary for normal development or tissue maintenance.

Necroptosis is a regulated form of cell death that is typically initiated when apoptosis is blocked. It begins with ligand binding to cell surface receptors that activate receptor-interacting serine/threonine-protein kinase 1 (RIPK1), which then forms a complex with RIPK3, termed the necrosome. Within this complex, RIPK3 undergoes autophosphorylation, a critical step that stabilizes its active conformation and facilitates subsequent phosphorylation of downstream targets. Then, RIPK3 phosphorylates mixed lineage kinase domain-like protein (MLKL), which oligomerizes and disrupts the plasma membrane, leading to cell rupture. RIPK3 can also be activated independently of RIPK1 through sensors such as Z-DNA-binding protein 1 (ZBP1) or Toll-like receptor adaptors like TRIF, further emphasizing its central role in the necroptotic pathway. Because RIPK3 integrates multiple upstream signals and directly governs MLKL

activation, it serves as the indispensable executioner of necroptosis. This pivotal position makes RIPK3 a promising therapeutic target to inhibit necroptosis (Su, 2025).

The RIPK3 inhibitors developed by GlaxoSmithKline—GSK'840, GSK'843, and GSK'872—exhibited high affinity and robust inhibition of RIPK3 *in vitro* but failed to block necroptosis in cellular models. This gap underscores the incomplete understanding of intracellular mechanisms regulating RIPK3 and MLKL activation. Recently, our laboratory developed a novel RIPK3 inhibitor, UH15-38, which shows potent suppression of necroptosis in both cell-based assays and *in vivo* models. (The supporting data are presented in the following Results section.)

The availability of this potent drug provides our lab with new opportunities to investigate the previously unexplored pathways that may still exist in RIPK3 and necroptosis.

Experimental Approach

Current evidence suggests that activation of MLKL by phosphorylation by RIPK3 is crucial for the activation of necroptosis. However, the specific phosphorylation sites on RIPK3 that mediate this activation remain poorly understood. Among the known sites, T224 and S227 are the best-characterized autophosphorylation sites. Blocking phosphorylation at these sites inhibits necroptosis, but this does not prevent the activation of RIPK3's kinase activity itself. Instead, T224/S227 phosphorylation appears to function by facilitating the interaction with MLKL following RIPK3 activation (Chen et al., 2013). This is supported by findings showing that these residues lie outside the activation loop, which is the canonical region where autophosphorylation typically regulates kinase activation (Reinhardt & Leonard, 2023).

Consistently, our recently developed inhibitor, UH15-38, effectively blocks necroptosis without inhibiting phosphorylation at T224/S227, indicating that these sites are not sufficient for RIPK3 to activate MLKL. Our evidence instead indicates the presence of additional autophosphorylation sites that may be mediating this process. Despite the importance of RIPK3 autophosphorylation in necroptosis, few studies have addressed which phosphorylation events directly regulate RIPK3 activation. Given that UH15-38 blocks RIPK3 from activating MLKL without impeding the previously reported autophosphorylation sites of T224/S227, we will utilize it as a molecular probe to dissect the critical mechanistic steps of RIPK3 activation in necroptosis and to understand the basis of UH15-38's efficacy both *in vitro* and in cellular models.

Hypothesis

I propose that RIPK3 activation depends on autophosphorylation events within its activation loop that occur specifically in a cellular context. UH15-38 blocks these critical phosphorylation events, thereby preventing full RIPK3 activation and necroptosis—an inhibition mechanism distinct from previously characterized sites like T224/S227 needed for binding MLKL, and from earlier GSK inhibitors.

Impact

Necroptosis is not a preferred mode of cell death, as its activation is linked to inflammatory and neurodegenerative diseases. A deeper understanding of the key regulatory steps underlying MLKL activation, which drives necroptosis, is essential for advancing knowledge of this pathway. Previous inhibitors developed by GlaxoSmithKline showed efficacy only *in vitro* using recombinant proteins, but failed in cellular systems. In contrast, our

compound UH15-38 demonstrates robust activity in both cellular and in vivo settings. By continuing to dissect the mechanisms of necroptosis, we can lay the groundwork for the development of more efficient therapeutics that can be administered directly in living organisms to block this pathway and mitigate downstream disease.

Methodology Used

- **Western blotting** is a method for detecting proteins that integrates gel electrophoresis, membrane transfer, and antibody-based recognition. Proteins are first separated by size using SDS-PAGE and then transferred onto a nitrocellulose or PVDF membrane, where they become immobilized. Detection is achieved through a specific primary antibody paired with a labeled secondary antibody, and the signal is typically visualized by chemiluminescence. This technique not only verifies the presence of a protein but also reveals its molecular weight, relative abundance, and post-translational modifications such as phosphorylation (Mahmood & Yang, 2012).
- **Immunoprecipitation with magnetic beads** is employed to isolate a specific protein from the complex mixture produced by cells. The beads, coated with a specific antibody, bind to the target protein in the cell lysate during incubation. Once binding occurs, the bead-protein-antibody complexes are separated from the solution using a magnet. Subsequent washing steps remove nonspecific proteins, and the target protein is finally eluted from the beads for downstream analyses such as Western blotting or mass spectrometry.
- **Viral-mediated gene delivery combined with antibiotic selection** is used in this research to allow the precise introduction of mutations into cells for the desired

necroptosis pathway mutation and its autophosphorylation sites. In this approach, a virus is engineered to carry the target gene containing the desired mutation along with an antibiotic resistance marker. Upon infection, the virus delivers both the mutated gene and the resistance gene into host cells, integrating into the genome. After infection, cells are cultured in media containing the corresponding antibiotic, which eliminates cells that did not receive the viral construct. Only those cells that have incorporated both the mutation and the resistance gene survive, resulting in a purified population suitable for downstream analyses.

Results

As expected, the *in vitro* kinase assay showed that UH15-38 and GSK'872 have comparable potency in inhibiting phosphorylation of RIPK3 and MLKL. This result validates our preliminary findings and demonstrates that UH15-38 and GSK'872 are equally effective at blocking necroptosis *in vitro*. In Figure 1B, the western blot reveals that treatment with 5 μM of either UH15-38 or GSK'872 nearly abolished phosphorylated RIPK3, while phosphorylated MLKL was completely eliminated.

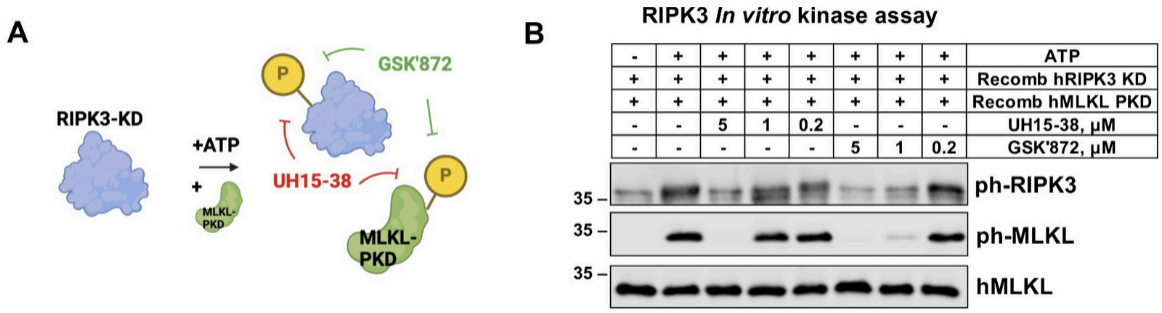

Figure 1: UH15-38 and GSK'872 have comparable potency against MLKL phosphorylation in vitro.

This effect was further evaluated in a cellular context. As shown in Figure 2A, treatment of HeLa RIPK3 cells with the necroptosis inducer TIS (TNFα + IDN-6556 + SM-164) reduced cell viability to ~40%. Co-treatment with UH15-38 significantly increased viability to ~90%, whereas GSK'872 afforded only a modest protective effect (~60% viability). These findings indicate that UH15-38 is a more potent inhibitor of necroptosis than GSK'872.

To investigate the underlying mechanism, RIPK3 complexes were immunoprecipitated using FLAG-tagged RIPK3 as illustrated in Figure 2B, and subsequently analyzed by immunoblotting (Figure 2C). UH15-38 treatment markedly reduced RIPK3 phosphorylation as well as downstream MLKL phosphorylation, consistent with its strong protective effect on cell viability. In addition, RIPK3 immunoprecipitated from UH15-38–treated cells exhibited increased electrophoretic mobility (yellow arrow), relative to untreated or GSK'872-treated samples (red and orange arrows, respectively), suggesting differences in phosphorylation state.

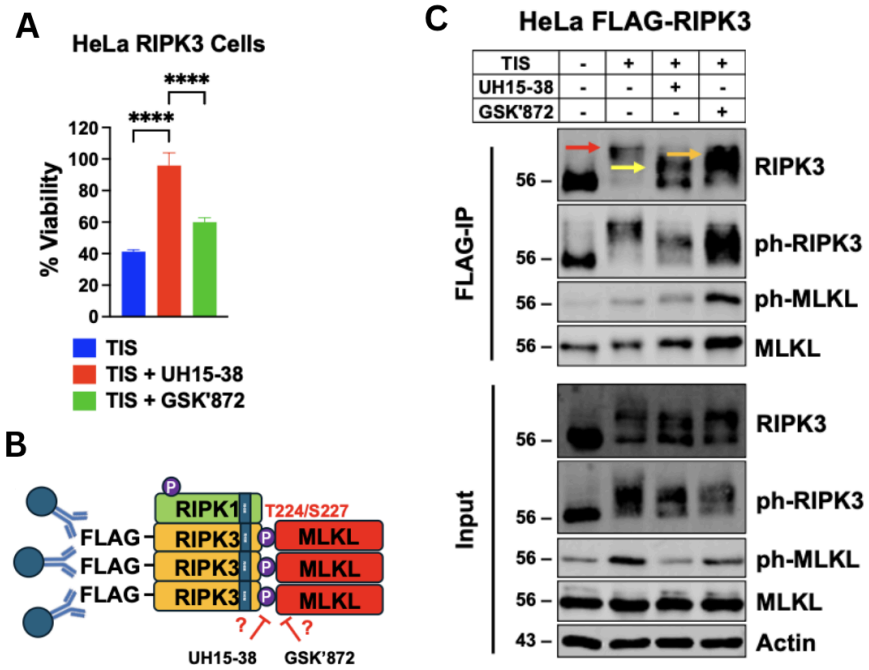

Figure 2: (A) FLAG-RIPK3 expression was induced with doxycycline for 6 hr, followed by TIS and 2.5 μM inhibitor treatment for 24 hr; cell viability was determined using the CellTiter-Glo assay. (B) Diagram of the FLAG-IP workflow. (C) Cells were treated as in (A) for 4 hr, after which FLAG-IP was performed and samples were examined by immunoblotting.

To further investigate how UH15-38 functions within cells, we examined its effects on the necrosome complex by immunoprecipitating FLAG-tagged MLKL from MEFs stimulated with TIS and treated with varying concentrations of the inhibitor. At 10 μM, UH15-38 completely abolished phosphorylation of both MLKL and RIPK3. In contrast, at 1 μM, UH15-38 effectively blocked MLKL phosphorylation but not RIPK3 phosphorylation. Again, here interestingly, under these conditions phosphorylated RIPK3 exhibited increased electrophoretic mobility on SDS-PAGE. Because protein migration on SDS-PAGE can be influenced by phosphorylation, we treated the immunoprecipitates with λ-phosphatase, an enzyme that removes phosphate groups from proteins. Following this treatment, the altered migration of RIPK3 was no longer observed, confirming that the mobility shift was phosphorylation-dependent. These results indicate that UH15-38 interferes with specific RIPK3 autophosphorylation sites, thereby altering its phosphorylation pattern while still preventing MLKL activation.

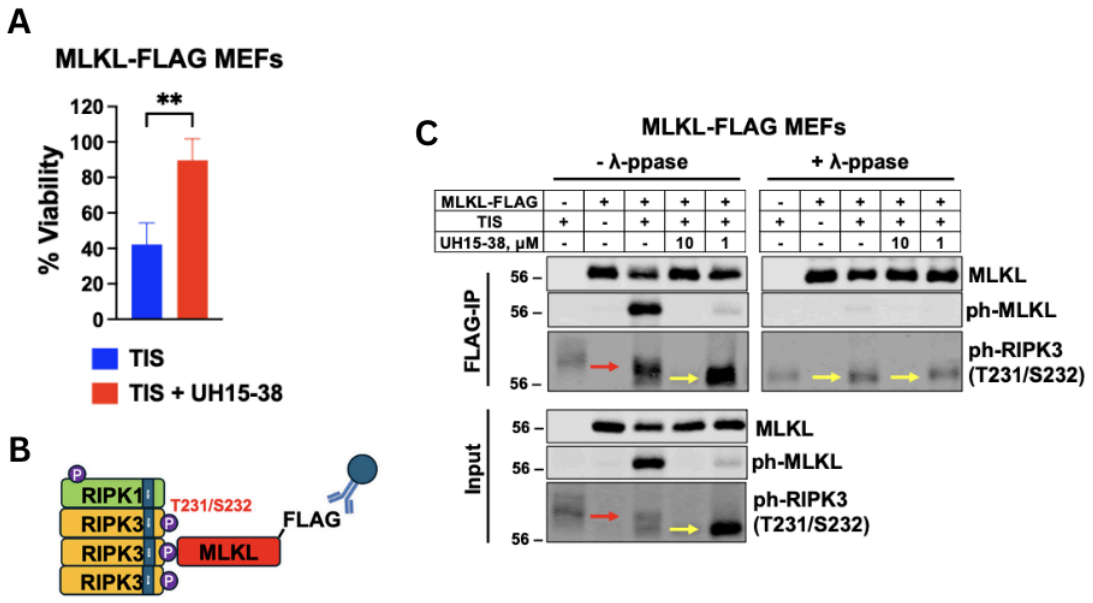

Figure 3: UH15-38 differentially regulates MLKL and RIPK3 phosphorylation.
At 10 μM, UH15-38 abolished phosphorylation of both MLKL and RIPK3, while at 1 μM it selectively blocked MLKL phosphorylation but altered RIPK3 phosphorylation, as shown by a λ-phosphatase–sensitive mobility shift.

Next, we wished to identify the additional RIPK3 phosphorylation sites responsible for the electromobility effect on SDS-PAGE that coincide with the inhibition of necroptosis. To study this, we aimed to establish a strategy that would increase our ability to recover phosphorylated RIPK3 for mass spectrometry analysis. A recently published paper discovered that a mutant form of MLKL prevents MLKL from forming oligomers that kill the cell without affecting any upstream steps (Ros, 2025) . For this purpose, we generated an MLKL mutant (L450R/S454R) that is defective in oligomerization in our cells. As expected, this mutant was unable to execute necroptosis. Interestingly, we observed that cells expressing MLKL L450R/S454R displayed a pronounced accumulation of phosphorylated RIPK3, visible as a slower-migrating band on SDS-PAGE. This suggests that when MLKL oligomerization is blocked, RIPK3 continues to undergo phosphorylation and accumulates in its phosphorylated state. We therefore reasoned that this mutant system could serve as a useful tool to enrich for phosphorylated RIPK3 in cells, thereby improving the likelihood of identifying novel RIPK3 phosphorylation sites by mass spectrometry.

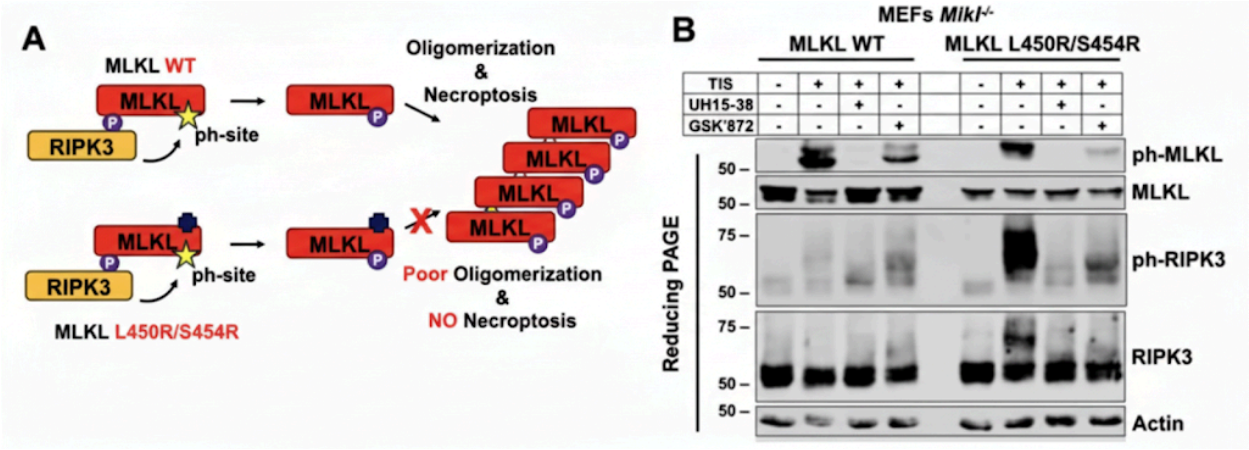

Figure 4: Inhibition of MLKL oligomerization leads to the accumulation of phosphorylated RIPK3.

We took advantage of the MLKL oligomerization mutant to enrich for the hyper-phosphorylated RIPK3. To have a viable system for purifying RIPK3 from cells, we expressed HA-tagged RIPK3 and performed HA immunoprecipitation from cells treated with TIS in the presence or absence of UH15-38. This strategy efficiently enriched phosphorylated RIPK3, as seen in the immunoblots, providing a strong input for mass spectrometry.

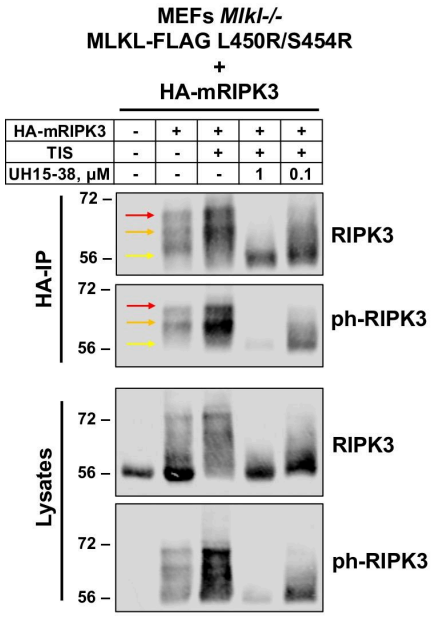

Figure 5: HA immunoprecipitation of RIPK3 in MLKL oligomerization-deficient cells enables efficient recovery of phosphorylated RIPK3.

Future Directions

To directly map RIPK3 phosphorylation sites targeted by UH15-38, I will employ mass spectrometry. Based on the identified sites, I will generate RIPK3 mutants with loss-of-function substitutions to define their specific contributions through mutagenesis using techniques such as PCR and Gibson assembly cloning, followed by lentiviral transduction. In the long term, this approach will allow me to pinpoint phosphorylation sites uniquely sensitive to UH15-38, clarify their roles in necroptotic signaling, and identify novel biomarkers of RIPK3 activation. I hope to continue this project throughout my undergraduate years at Tufts, potentially developing it further as the basis of my senior thesis.

References

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