Combinatorial use of VHL and KEAP1-based PROTACs reveals unexpected synergy and hook effect relief

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ABSTRACT

Proteolysis-targeting chimeras (PROTACs) are bifunctional molecules bridging a target protein with an E3 ubiquitin ligase, promoting its ubiquitylation and degradation. Unlike traditional inhibitors, PROTACs exploit transient interactions, enabling the selective degradation of "undruggable" proteins, thereby expanding the landscape of druggable targets. However, PROTACs are not without limitations. The "hook effect"—a phenomenon where high PROTAC concentrations reduce efficacy due to inactive binary complex formation—compromises their clinical utility. Additionally, single PROTACs may fail to achieve complete degradation due to insufficient polyubiquitination or suboptimal E3 ligase recruitment, limiting their therapeutic efficacy. These challenges highlight the need for strategies to enhance PROTAC performance and so therapeutic applicability. In this study, we introduce a novel dual-PROTAC strategy that recruits two distinct E3 ligases, such as KEAP1 and VHL, to cooperatively degrade KRAS(G12D) and Androgen Receptor (AR). Our data demonstrate that combining KEAP1- and VHL-recruiting PROTACs synergistically enhances target degradation, reduces the required dosing, and minimizes off-target effects. Notably, KEAP1 recruitment offers the unique advantage to favor polyubiquitin chain elongation, thereby improving proteasomal recognition. In conclusion, a dual-E3 ligase approach represents a promising avenue for optimizing PROTAC-based therapeutics.

INTRODUCTION

The development of bifunctional degraders (PROTACs) that chemically link target proteins to E3 ubiquitin ligases has significantly advanced the targeting of undruggable proteins including KRAS(G12D). PROTACs induce the formation of a ternary complex between the drug, target protein, and E3 ligase, leading to ubiquitination and proteasomal degradation. The dynamic nature of ternary complex formation affects degradation efficiency, with factors such as binding kinetics, thermodynamics, and target engagement influencing efficacy. At high concentrations, PROTAC activity decreases due to excessive formation of binary complexes, preventing productive ternary complex formation and leading to suboptimal degradation, a phenomenon known as the hook effect. Optimizing drug concentration, tissue penetration, and tumor microenvironment factors are crucial for maximizing PROTAC efficacy in clinical applications.

Hijacking the Ubiquitin-Proteasome System (UPS), using small molecules for targeted protein degradation

PROTAC-mediated ternary complex formation and Hook effect

ST-00974 is VHL-based PROTAC which promoted degradation of KRAS(G12D)

Figure 1 (**A**) Chemical structures of compound ST-00974. (**B**) Western blot analysis of AsPC1 cells treated with ST-00974 in presence or absence of Bortezomib or MLN4924. (**D**) Volcano plot representing the $\log_2$(fold change) over the -$\log_{10}$(Padj) of the differentially expressed genes (ST-00974/DMSO) for AsPC1 cells revealed by RNA-seq. **(E)** Dot plot showing the GSEA for the transcriptomes in panel D. **(F)** Heat map showing the relative expression of RAS and IFN-signature genes using the normalized counts from the transcriptome analysis in panel D.

KEAP1-based KRAS(G12D) PROTACs are not effective degraders as single agent

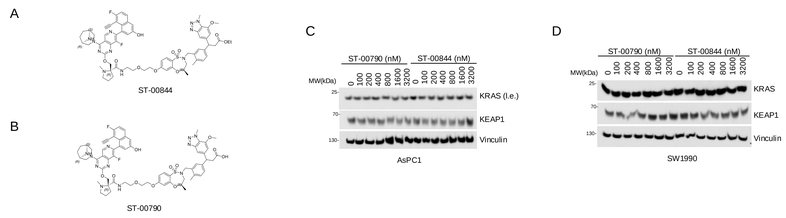

Figure 2 (**A**) Chemical structure of compounds ST-00844 and ST-00790. **(C)** Western blot analysis of AsPC1 cells treated with increasing doses of ST-00790 and ST-00844. **(D)** Western blot analysis of SW1990 cells treated with increasing doses of ST-00790 and ST-00844.

KEAP1-based PROTAC synergizes with and releases the hook effect induced by a VHL-PROTAC targeting KRAS(G12D)

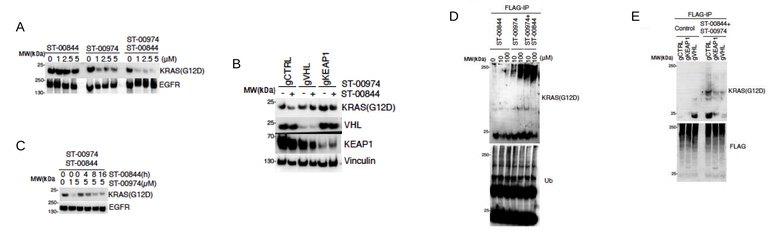

Figure 3 (**A**) (**A**) Western blot analysis of AsPC1 cells treated with increasing doses of ST-00844 or ST-00974 or their combination. (**B**) Western blot analysis of AsPC1 cells stably expressing sgRNAs targeting VHL or KEAP1, treated with ST-00790 and ST-00844. **(C)** Western blot analysis of AsPC1 cells were treated with ST-00974 and subsequently treated with ST-00844 for the indicated time. **(D)** Cell lysate Ubiquitination: AsPC1 cell lysates were incubated with an ATP regeneration system, E1 enzyme, E2 enzyme, and FLAG-ubiquitin, in the presence of the indicated drug concentrations. Reaction mixtures were incubated at 30°C for 2 hours. After incubation, samples were denatured and immunoprecipitated with anti-FLAG antibody. Western blot analysis was performed to detect the KRAS(G12D) ubiquitination. **(E)** AsPC1 cells stably expressing sgRNAs targeting VHL or KEAP1, or a control sgRNA were collected and cell lysate ubiquitination assay was performed as described in panel D.

KEAP1-based PROTAC enhances the ubiquitylation of KRAS(G12D) pre-ubiquitinated by the VHL-PROTAC complex

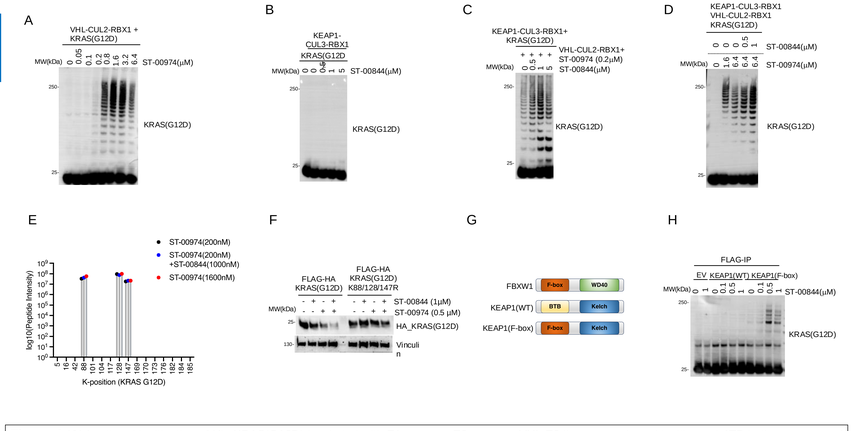

Figure 4 (**A**) In vitro ubiquitination of His-KRAS(G12D) incubated with E1 enzyme, E2 enzyme, VHL E3 ligase complex, ubiquitin, and ATP in the presence of increasing doses of ST-00974. **(B)** In vitro ubiquitination of His-KRAS(G12D) incubated with E1 enzyme, E2 enzyme, KEAP1 E3 ligase complex, ubiquitin, and ATP in the presence increasing doses of ST-00844, with or without 200 nM of ST-00974 (VHL E3 ligase complex. **(C)** In vitro ubiquitination assay of Recombinant His-KRAS(G12D) incubated with E1 enzyme, E2 enzyme, KEAP1 and VHL E3 ligase complexes, ubiquitin, and ATP in the presence of different doses of ST-00844 and ST-00974, as indicated. **(D)** Mass spectrometry analysis of His-KRAS(G12D) upon In vitro ubiquitination assay and data represents the log10 peptides intensity for the indicated sites. **(E)** Western blot analysis of AsPC1 cells stably expressing HA-tagged KRAS(G12D) or KRAS(G12D) K88/128/147R mutant, treated with ST-00844, ST-00974, or their combination. **(F)** HEK-293 T cells were transfected with FLAG-HA-TurboID_KRAS(G12D) for 36h. Cells were lysed with NP40 buffer, and the cell lysate were immunoprecipitated with anti-FLAG antibody in the presence of ST-00844 or ST-00974. Input and immunoprecitates were probed with indicated antibodies. **(G)** Schematic representation of β-TrCP1, KEAP1, and chimeric KEAP1(F-box) domain structures **(H)** In vitro ubiquitination His-KRAS(G12D) incubated with E1 enzyme, E2 enzyme, immunopurified KEAP1 and KEAP1(F-box) E3 ligase complexes, ubiquitin, and ATP in the presence of increasing doses of ST-00844.

VHL and KEAP1-based PROTACs synergistically inhibit the proliferation of KRAS(G12D) positive pancreatic cancer cells

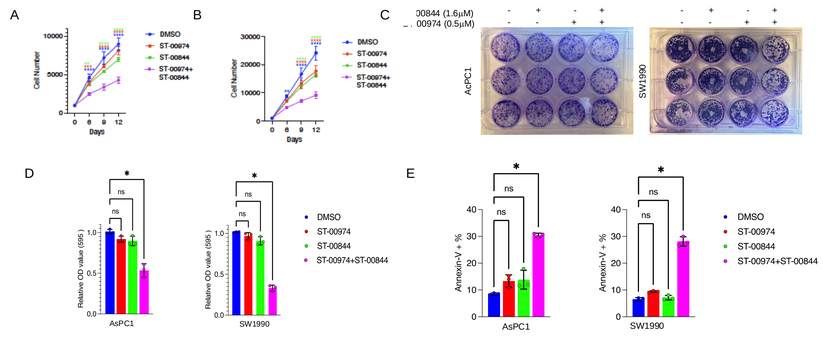

Figure 5 (**A**) Cell Viability of AsPC1 cells treated with ST-00844, ST-00974, or their combination in the presence of 2.5 μM of Zosuquidar. **(B)** Cell Viability of SW1990 cells treated with ST-00844, ST-00974, or their combination in the presence of 2.5 μM of Zosuquidar. **(C)** Colony formation of AsPC1 or SW1990 cells treated with ST-00844, ST-00974, or their combination in the presence of 2.5 μM of Zosuquidar. **(D)** Colony quantification measured by a microplate reader at OD 570 nm. **(E)** AsPC1 and SW1990 cells treated with ST-00844, ST-00974, or their combination in the presence of 2.5 μM of Zosuquidar for 9 days. Cells stained for Annexin-V for quantification of apoptosis induction.

Synergy of VHL and KEAP1 based PROTACs on Androgen Receptor (AR) in prostate cancer

Figure 6 ((**A**) Chemical structures of VHL- and KEAP1-based AR PROTACs. (**B**)) Western blot analysis of 22Rv1 cells treated with increasing doses of ST-01584 or ST-01570. **(C)**) Western blot analysis of 22Rv1 cells treated with increasing doses of ST-01567 or ST-01549. **(D)**) Western blot analysis of 22Rv1 cells treated with increasing doses of ST-01549, ARCC4 or ARV-110. **(E)**) Western blot analysis of 22Rv1 cells treated with different doses of KEAP1-based AR PROTACs, ARCC4 or their combination. **(F)**) Western blot analysis of 22Rv1 cells treated with different doses of ARCC4 for 6h and then with ST-01549. **(G)** Colony formation of 22Rv1 or LnCAP cells treated with ST-01549, ARCC4, or their combination in the presence of 2.5 μM of Zosuquidar. **(H)** Colony quantification measured by a microplate reader at OD 570 nm.

CONCLUSION / FUTURE DIRECTIONS

- KEAP1 based PROTACs are weak degraders as opposed to VHL-based PROTACs.
- VHL-based PROTACs display the classic “hook” effect on target degradation.
- KEAP1 based PROTACs synergize with VHL-based PROTAC in promoting KRAS(G12D) and AR degradation.
- KEAP1 based PROTACs release the hook effect of VHL-based PROTACs.
- KEAP1 and VHL based PROTACs synergize in inhibiting Pancreatic cancer cells proliferation and induce apoptosis by targeting KRAS(G12D).
- KEAP1 and VHL based PROTACs synergize in inhibiting Prostate cancer cells proliferation by targeting AR.
- Further research is needed to advance dual PROTACs into preclinical mouse models of pancreatic and/or prostate cancer.
- Assess other E3 ligases for for their ability to facilitate ubiquitin chain elongation.
- Explore the cooperative roles of different E3 ligases in targeted protein degradation.

PROPOSED MODEL

KEAP1-based PROTACs are ineffective as single agents but synergize with VHL-based PROTACs by promoting efficient ubiquitin chain elongation