Title: Targeted Degradation of Oncogenic KRAS\textsuperscript{G12C} by VHL-recruiting PROTACs

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Abstract:

KRAS is mutated in \textasciitilde 20\% of human cancers and is one of the most sought-after targets for pharmacological modulation, despite having historically been considered “undruggable.” The discovery of potent covalent inhibitors of the KRAS\textsuperscript{G12C} mutant in recent years has sparked a new wave of interest in small molecules targeting KRAS. While these inhibitors have shown promise in the clinic, we wanted to explore PROTAC-mediated degradation as a complementary strategy to modulate mutant KRAS. Herein, we report the development of LC-2, the first PROTAC capable of degrading endogenous KRAS\textsuperscript{G12C}. LC-2 covalently binds KRAS\textsuperscript{G12C} with a MRTX849 warhead and recruits the E3 ligase VHL, inducing rapid and sustained KRAS\textsuperscript{G12C} degradation leading to suppression of MAPK signaling in both homozygous and heterozygous KRAS\textsuperscript{G12C} cell lines. LC-2 demonstrates that PROTAC-mediated degradation is a viable option for attenuating oncogenic KRAS levels and downstream signaling in cancer cells.

Introduction:

The Kirsten rat sarcoma viral oncogene homolog (KRAS) gene is one of the most frequently mutated oncogenes in cancer\textsuperscript{1-3}. KRAS encodes a small, membrane bound GTPase that relays signals from receptor tyrosine kinases (RTKs), promoting cell proliferation, cell differentiation or cell death\textsuperscript{4-5}. In normal cells, KRAS functions as a molecular switch, cycling between an inactive, GDP-bound “off” state and an active, GTP-bound “on” state\textsuperscript{4,6}. This switch is tightly regulated by guanine nucleotide exchange factor (GEF) proteins, which exchange GDP for GTP, and GTPase-activating proteins (GAPs), which enhance the intrinsically slow GTPase activity of KRAS\textsuperscript{7-9}. GEF and GAP effector proteins bind at one or both of two shallow binding pockets on KRAS termed switch I (residues 30-38) and switch II (residues 59-76), the conformations of which change dramatically
between GDP- and GTP-bound states. Somatic KRAS mutations attenuate the GAP-mediated enzymatic activity of the protein, resulting in accumulation of GTP-bound, active KRAS and hyperactivation of downstream signaling, which leads to uncontrolled cell proliferation. Despite its prevalence in cancer and many years of extensive research efforts, mutant KRAS has remained a challenging therapeutic target given the scarcity of traditional druggable pockets on its surface.

The KRAS p.G12C mutation is highly prevalent in lung adenocarcinoma (LUAD). KRAS G12C mutants make up over 50% of all KRAS mutant LUAD tumors (13% of total LUAD tumors). Additionally, 3% of colorectal cancers and 1% of all other solid tumors express KRAS G12C. This mutation greatly reduces KRAS's intrinsic GTPase activity, allowing for the accumulation of GTP-bound, active KRAS. Recent advances, initially led by the Shokat group, have identified molecules that covalently and selectively bind the mutated cysteine of KRAS G12C. These compounds induce a novel, drug-like pocket within the KRAS switch II region. Optimization of the electrophiles responsible for conjugating the cysteine as well as the molecular interactions within the drug-induced pocket have led to the development of orally bioavailable KRAS G12C inhibitors. ARS-1620/ARS-3248, AMG510, and MRTX849, developed by Wellspring, Amgen, and Mirati Therapeutics respectively, have been shown to potently inhibit KRAS G12C activity in vitro and in vivo. In addition, ARS-3248, AMG510 and MRTX849 have entered phase I clinical trials and have shown promising results. However, despite this success, rapid adaptive resistance and MAPK signaling reactivation after inhibitor treatment have already been reported. Thus, the development of complementary therapeutic strategies could help realize the full potential of targeting KRAS mutants for cancer treatment.

PROteolysis TArgeting Chimeras (PROTACs) have emerged as a new and promising modality in drug discovery. These bifunctional molecules simultaneously engage a protein of interest (POI) and an E3 ligase, forming a ternary complex, enabling the E3 ligase to ubiquitinate the POI on proximal lysine residues. The ubiquitinated POI is subsequently recognized and degraded by the 26S proteasome. A major advantage of target degradation is the elimination of scaffolding roles that are not typically attenuated by traditional small-molecule inhibitors. PROTACs incorporating ARS-1620 and the cereblon E3 ligase ligand thalidomide were recently published by the Gray group. These molecules engage KRAS G12C and degrade an artificial GFP-KRAS G12C fusion protein, but were unable to degrade endogenous KRAS. Herein, we report the development of the first-in-class endogenous KRAS G12C degrader, LC-2, which combines MRTX849 with a VHL E3 ligase ligand. We observe rapid degradation through a bona fide PROTAC mechanism in both homozygous and heterozygous KRAS G12C-expressing cells. Acute and sustained degradation of
KRAS\textsuperscript{G12C} in multiple cancer cell lines renders LC-2 a valuable tool compound to interrogate KRAS biology and represents a significant step towards the development of PROTAC-based candidate therapeutics that function by inducing oncogenic KRAS degradation.

**Results:**

MRTX849 based VHL-recruiting PROTACs engage and degrade endogenous KRAS\textsuperscript{G12C} in homozygous and heterozygous mutant cell lines.

![Chemical structures](image)

**Figure 1:** MRTX849-VHL PROTACs engage and degrade endogenous KRAS\textsuperscript{G12C} in NCI-H2030 cells: A) Chemical structures of MRTX849, LC-1 (inactive PROTAC), LC-2 (active PROTAC), and LC-2 Epimer. B) LC-1 engages KRASG12C in a dose dependent manner. Quantitation on the right. C) LC-2 degrades KRASG12C in a dose dependent manner. Quantitation on the right. Quantified data represents mean ± SD. Not Significant (N.S.); * p < 0.05; ** p < 0.01; **** p < 0.001

In view of its promising Phase I clinical data and synthetic tractability, we chose MRTX849 (MRTX; Figure 1A) as a starting point to design KRAS-targeting PROTACs. Docking of MRTX into the “switch II” pocket of KRAS\textsuperscript{G12C} reveals the pyrrolidine group to be solvent exposed (PDB: 5V9U; SI Figure 1A)\textsuperscript{19}. To avoid introducing another stereocenter at the 2,3 or 4 position of the pyrrolidine and further complicating our synthetic route, we decided to build linkers from the N-methyl moiety of the pyrrolidine. We saw our first evidence of KRAS engagement with LC-1 (Figure 1A and B). When NCI-H2030 cells were treated with increasing concentrations of LC-1 for 24 hours we observed a clear band shift at 1, 2.5, 10, and 25 μM, indicating the presence of PROTAC-conjugated KRAS (Figure 1B). However, only a small, non-significant reduction in KRAS levels was observed. Therefore, these data indicate that LC-1 can engage KRAS\textsuperscript{G12C}, but does not
efficiently degrade the protein. As a result, **LC-1** was subsequently used as a positive control for KRAS engagement during our PROTAC screen.

| Cell Line  | KRAS<sup>G12C</sup> Genotype | DC<sub>50</sub> (μM) | D<sub>max</sub> (%) | Mirati Sensitivity<sup>21</sup> |
|------------|-------------------------------|---------------------|-------------------|-----------------------------|
| NCI-H2030  | Homozygous                    | 0.59 ± 0.2          | > 75              | +                           |
| MIA PaCa2  | Homozygous                    | 0.32 ± 0.08         | > 75              | ++                          |
| SW1573     | Homozygous                    | 0.76 ± 0.3          | > 85              | -                           |
| NCI-H23    | Heterozygous                  | 0.25 ± 0.08         | > 90              | +++                         |
| NCI-H358   | Heterozygous                  | 0.52 ± 0.3          | > 40              | +++                         |

Table 1. LC-2 induces degradation of endogenous KRAS<sup>G12C</sup> in multiple KRAS mutant cancer cell lines: PROTAC activity in a panel of KRAS<sup>G12C</sup> cancer cell lines. DC50 at which 50% of the maximal degradation (Dmax) is reached.

One major liability of **LC-1** is the presence of a hydrolysable amide within the linker. To address this liability, the linkers of subsequent PROTACs were extended directly from the pyrrolidine ring nitrogen. We screened a small library of PROTACs with linker lengths several atoms shorter and longer than **LC-1**, and from this screen, we identified **LC-2** as the most potent KRAS<sup>G12C</sup>-degrading PROTAC (Figure 1A). **LC-2** induced maximal degradation of endogenous KRAS<sup>G12C</sup> at concentrations as low as 2.5 μM with a D<sub>max</sub> of >75% and a DC<sub>50</sub> of 0.59 ± 0.2 μM in NCI-H2030 cells (Figure 1C). At 10 μM **LC-2**, a KRAS<sup>G12C</sup> band running at the same molecular weight as **LC-1**-modified KRAS<sup>G12C</sup> was observed. The emergence of this undegraded higher molecular weight band suggests the start of a “hook-effect” at high **LC-2** concentrations. The “hook-effect” is a hallmark of PROTACs, whereby at high drug concentrations, the formation of unproductive dimers with target or with E3 ligase outcompete formation of the ternary complex necessary for degradation<sup>40</sup>.

**MRTX** is known to be selective for mutant KRAS<sup>G12C</sup> over other KRAS mutants<sup>21</sup>. To explore the specificity of **LC-2**, KRAS degradation was examined in HCT 116 cells, which harbor a heterozygous KRAS<sup>G13D</sup> mutation. No engagement or degradation of KRAS<sup>G13D</sup> was observed in the presence of **LC-2** up to 10 μM (SI Figure 1B). These data further suggest that **LC-2** selectively engages and degrades mutant KRAS<sup>G12C</sup> protein.

In addition, we tested **LC-2** in 5 different KRAS<sup>G12C</sup> cell lines and observed DC<sub>50</sub> values between 0.25 and 0.76 μM as well as D<sub>max</sub> values ranging from >75-90% (Table 1). **LC-2** can degrade
mutant KRAS in both homozygous and heterozygous cell lines with varying sensitivities to MRTX$^{21}$. We observed >50% degradation in NCI-H23 cells, which are heterozygous. Theoretically, since these cells carry one wild type and one mutant KRAS$^{G12c}$ allele, one would expect a maximum of 50% degradation if expression were equal, as we see for NCI-H358 cells (SI Figure 2A). However, in siRNA knockdown experiments using KRAS$^{G12C}$ specific siRNA, nearly complete loss of KRAS is observed for NCI-H23 cells, which is consistent with the degradation we observe with LC-2. Cumulatively, these data show that MRTX-based, VHL-recruiting PROTACs can engage and degrade KRAS$^{G12C}$ in multiple cancer cell lines.

LC-2 induced KRAS$^{G12C}$ degradation occurs via a bona fide PROTAC mechanism.

Figure 2: Degradation of endogenous KRASG12C is via a PROTAC mechanism. A) LC-2 Epimer does not induce KRASG12C degradation at 2.5 μM and LC-2 induced degradation is rescued by VHL ligand competition, proteasome inhibition with epoxomicin (Epox), and neddylation inhibition with MLN4924 (MLN), in NCI-H2030 cells. Quantitation is below. B) Inhibition of neddylation, but not inhibition of lysosomal acidification, rescues LC-2 induced KRASG12C degradation in NCI-H23 cells. Quantitation is below. Quantified data represents mean ± SD. Not Significant (N.S.); *** p < 0.005

The hydroxy proline moiety of the VHL ligand confers binding to the E3 ligase, while inversion of the absolute stereochemistry of the 4-hydroxy proline moiety abrogates VHL binding$^{39}$. Therefore, we synthesized LC-2 Epimer (Figure 1A) as a physicochemically-matched negative control.
molecule that is unable to recruit VHL. When NCI-H2030 cells were treated with 2.5 μM LC-2 Epimer for 4 hours, only KRAS engagement was observed, whereas 2.5 μM LC-2 induced significant degradation (~65%; Figure 2A).

PROTACs target proteins for degradation via the proteasome by facilitating their ubiquitination, which is dependent on the formation of a ternary complex between the POI, PROTAC and the E3 ligase – in this case, VHL. Since excess VHL ligand inhibits ternary complex formation, we performed competition experiments in NCI-H2030 cells that were pre-treated for 1 hour with molar excess of VHL ligand before being treated with 2.5 μM LC-2. Competition of LC-2 with VHL ligand rescued KRAS\(^{G12C}\) levels (Figure 2A) by preventing PROTAC engagement with VHL. However, the higher molecular weight KRAS\(^{G12C}\) band observed upon LC-2 treatment demonstrates that the PROTAC was nevertheless still able to engage KRAS\(^{G12C}\).

Neddylation of CUL2, a VHL adaptor protein, is necessary for proper assembly and function of the VHL E3 ligase complex\(^{42}\). To further investigate whether LC-2 induced degradation of KRAS\(^{G12C}\) occurs via a bona fide PROTAC mechanism, NCI-H2030 cells were treated with 1 μM of the neddylation inhibitor MLN4924 or 1 μM of the proteasome inhibitor epoxomicin, before being treated with 2.5 μM LC-2\(^{43-44}\). Both inhibitors rescued KRAS\(^{G12C}\) levels suggesting KRAS\(^{G12C}\) degradation by LC-2 is both proteasome- and neddylation-dependent (Figure 2A).

KRAS is tethered to the plasma membrane, and it is possible that monoubiquitination of KRAS\(^{G12C}\) could induce endocytosis and degradation of KRAS\(^{G12C}\) through the lysosomal pathway\(^{45}\). Therefore, we also tested whether bafilomycin A1 (BafA1), an inhibitor of lysosomal acidification, could rescue KRAS\(^{G12C}\) degradation\(^{46}\). Pre-treatment of NCI-H23 with BafA1 was unable to rescue LC-2 induced KRAS\(^{G12C}\) degradation, whereas neddylation inhibition again rescued KRAS degradation (Figure 2B). Taken together these data show that LC-2-induced KRAS\(^{G12C}\) degradation is dependent on ternary complex formation with VHL and a functioning ubiquitin proteasome system, but not dependent on the lysosome.

**LC-2 induce rapid and sustained KRAS\(^{G12C}\) degradation in multiple cancer cell lines**

To explore PROTAC-induced KRAS\(^{G12C}\) degradation kinetics, time course experiments were performed in NCI-H2030 cells and SW1573 cells using 2.5 μM LC-2 as the fixed concentration since it induced maximal degradation in all cell lines within 24 hours (Figure 1A and SI Figure 2). To distinguish between rates of target engagement and degradation, LC-2 Epimer was used as a negative control to monitor KRAS\(^{G12C}\) engagement. Quantitation of engagement was achieved by comparing the intensity of just the LC-2 Epimer modified band to the intensity of unbound KRAS in
DMSO treated samples (see Material and Methods). For NCI-H2030 cells, KRAS\textsuperscript{G12C} binding was seen as early as 1 hour for both LC-2 and LC-2 Epimer (Figure 3A). Maximal engagement and significant degradation occurred within 4 hours. Maximum degradation was reached by 8 hours in NCI-H2030 cells and persisted up to 24 hours. SW1573 cells showed faster kinetics with near maximal engagement at 1 hour. However, the degradation rate was slower than NCI-H2030 cells as maximal degradation was not observed until 12 hours (Figure 3B).

During our PROTAC screen, we observed that 0.1 μM of MRTX and 10 μM of LC-1 increased KRAS protein levels (Fig 1C). Although Hallin, et al. did not observe increased KRAS\textsuperscript{G12C} protein levels with MRTX, our data is consistent with previous observations made with the KRAS\textsuperscript{G12C} inhibitor ARS1620\textsuperscript{19, 21}. Therefore, we explored how longer treatments with LC-2 would affect KRAS\textsuperscript{G12C} levels. MIA PaCa-2, NCI-H23, and SW1573 cells were treated with 2.5 μM of LC-2 for 6,
24, 48, and 72 hours. In all three cell lines maximal KRAS degradation occurred within 24 hours and was sustained up to 72 hours (Figure 4A-B and SI Figure 3). LC-2 Epimer fully engaged KRAS\textsuperscript{G12C} in SW1573 cells, but did not decrease protein levels as expected (SI Figure 3). In NCI-H23 cells, KRAS\textsuperscript{G12C} began to rebound at 72 hours. Taken together these data show that LC-2 is capable of rapid and sustained KRAS\textsuperscript{G12C} degradation in both homozygous and heterozygous cell lines. The ability to overcome increased KRAS\textsuperscript{G12C} expression suggests that degradation could be more beneficial than inhibition for prolonged attenuation of downstream signaling as has been observed previously with BRD4 degraders\textsuperscript{47}.

**Figure 4:** Degradation of endogenous KRAS\textsuperscript{G12C} is sustained over 72 hours in multiple cancer cell lines. A) 72 hour time course in MIA PaCa-2 cells. Degradation occurs at 6 hours and is maintained up to 72 hours. Quantitation on the right. B) 72 hour time course in NCI-H23 cells. Degradation occurs within 6 hours, reaches a maximum at 24 hours, and begins to rebound by 72 hours. Quantitation on the right. Quantified data represents mean ± SD. Not Significant (N.S.); ** p < 0.01; *** p < 0.005; **** p <0.001

**LC-2 induced** KRAS\textsuperscript{G12C} degradation modulates Erk signaling in homozygous and heterozygous KRAS mutant cell lines

The ability of LC-2 to modulate Erk signaling was investigated in NCI-H2030 and NCI-H23 cells during a 24 hour dose response. In NCI-H2030 cells, pErk was detected and a dose-dependent decrease in signaling was observed (Figure 5A). In addition, total Erk levels were elevated in a
dose-dependent manner. NCI-H23 cells showed a similar dose-dependent decrease in pErk (Figure 5B). Additionally, total Erk levels were elevated in a dose-dependent manner.

![Effects of LC-2 on Erk Signaling NCI-H2030](image1)

![Effects of LC-2 on Erk Signaling NCI-H23](image2)

Figure 5: Degradation of endogenous KRASG12C modulates Erk signaling in homozygous and heterozygous KRASG12C cell lines. A) Degradation of KRASG12C in homozygous NCI-H2030 cells attenuates pErk in a dose dependent manner. Quantitation on the right. B) Degradation of KRASG12C in heterozygous NCI-H23 cells decreases pErk in a dose dependent manner. Quantitation on the right. For statistical analysis see Supplemental Tables 1 and 2. Quantified data represents mean ± SD.

Signaling kinetics were monitored during a 24 hour time course in MIA PaCa2, NCI-H23, and SW1573 cells treated with 2.5 μM LC-2. Modulation of Erk signaling by both MRTX and LC-2 occurs within 6 hours in MIA PaCa-2 and NCI-H23 cells (Figure 6A-B and SI Figure 4). pErk was suppressed by both compounds at 6 and 24 hours in each cell lines. In SW1573 cells, phosphorylated Erk was inhibited by 2.5 μM LC-2 between 1 and 4 hours, however pErk levels rebounded between 8 and 24 hours (SI Figure 4). Nonetheless, pErk levels were still significantly...
lower in LC-2 treated cells than DMSO treated cells at 24 hours. Total Erk was increased in LC-2 treated cells compared to DMSO at all time points indicating the initiation of a positive feedback loop upon KRAS<sup>G12C</sup> degradation and pErk inhibition. Taken together, these data show that LC-2-induced KRAS<sup>G12C</sup> degradation is capable of modulating downstream signaling and that differences in signaling between inhibition and degradation are cell line dependent.

Discussion:

![Figure 6: Effect of KRASG12C degradation and inhibition on Erk signaling over time. A) Inhibition and degradation of KRASG12C decreases pErk signaling at 6 and 24 hours in homozygous MIA PaCa-2 cells. Quantitation on the right. B) Inhibition and degradation of KRASG12C decreases pErk signaling at 6 and 24 hours in heterozygous NCI-H23. Quantitation on the right. For statistical analysis see Supplemental Tables 3 and 4. Quantified data represents mean ± SD.](image)

To our knowledge, this study is the first report of PROTAC-induced endogenous KRAS<sup>G12C</sup> degradation in cancer cells. Our PROTAC, LC-2, couples the covalent KRAS<sup>G12C</sup> inhibitor MRTX to the VHL ligand developed in our laboratory<sup>21, 39</sup>. VHL recruitment to KRAS<sup>G12C</sup> induces endogenous KRAS ubiquitination and degradation with DC<sub>50</sub> values ranging from 0.25 to 0.76 µM. We observe
rapid engagement, sustained KRAS degradation, and attenuated pErk signaling for up to 72 hours in several KRAS\textsuperscript{G12C} mutant cell lines. This tool compound will facilitate further exploration of how KRAS degradation influences downstream signaling and the viability of KRAS\textsuperscript{G12C} mutant cancer cells with more precise temporal control than nucleic acid-based knockdown methods.

This work is not the first attempt at degrading KRAS\textsuperscript{G12C}. Recently, Zeng \textit{et al.} were unsuccessful in degrading endogenous KRAS\textsuperscript{G12C} with 20 μM of \textit{XY-4-88} over 24 hours\textsuperscript{38}. That PROTAC was based on \textbf{ARS1620} and used thalidomide to recruit cereblon, whereas our active PROTAC, \textbf{LC-2}, is \textbf{MRTX}-based and recruits VHL. It has been our observation that differences in either constituent ligand of a PROTAC can significantly impact the efficacy and selectivity of target engagement\textsuperscript{31, 48}.

Further studies will focus on understanding the importance of the KRAS\textsuperscript{G12C} ligand, the recruited E3 ligase, or combination of the two factors in imparting \textbf{LC-2}'s activity. Conducting ternary complex assays by SPR and/or monitoring the ability of these compounds to induce ubiquitination by using tandem ubiquitin binding entity (TUBE) pulldowns followed by immunoblotting could address these questions\textsuperscript{49}.

With the availability of several new covalent inhibitors, kinome re-wiring in response to KRAS\textsuperscript{G12C} inhibition has been an active research area. It has been found that signaling attenuated by \textbf{MRTX}, AMG510, and ARS1620 returns to or exceeds basal levels between 24 and 72 hours\textsuperscript{21, 24-25}. This has been linked to the increased activity of several tyrosine kinases. To combat this acquired resistance, both targeted inhibition with FGFR inhibitors as well as pan-RTK inhibition with SHP2 inhibitors in combination with KRAS\textsuperscript{G12C} inhibition have been successfully used to reduce the recovery of inhibited signaling\textsuperscript{24}. These co-treatment regimens have also been shown to be more anti-proliferative \textit{in vitro} and \textit{in vivo} compared to RTK inhibition or KRAS\textsuperscript{G12C} inhibition alone\textsuperscript{24, 50}. It will be interesting to determine whether degradation alone can overcome Erk signaling reactivation and/or if combination of KRAS degradation with RTK inhibition could further enhance antiproliferative effects. In addition to the re-wiring of sensitive cells, there are known cell lines, such as SW1573 (used in this work) and NCI-H1792, that are inherently resistant to the anti-proliferative effects of KRAS\textsuperscript{G12C} inhibition. Recently, it was shown that siRNA mediated knockdown in these cells, but not KRAS\textsuperscript{G12C} inhibition, resulted in ~50% decreased cell viability\textsuperscript{50}. Therefore, it will be interesting to determine if KRAS\textsuperscript{G12C}-induced degradation of KRAS\textsuperscript{G12C} by \textbf{LC-2} is also similarly antiproliferative in these cell lines.

The ability to target KRAS with covalent inhibitors was itself a milestone in drug discovery. It showed that proteins formally deemed undruggable could be targeted with small molecules. Similarly, the results presented here demonstrate that endogenous KRAS\textsuperscript{G12C} can be degraded as
long as a suitable ligand is identified. While ligand development for other KRAS mutants continues, LC-2 can serve as a tool compound to investigate biology in the context of rapid KRAS G12C degradation, i.e., cell viability. One caveat with our lead compound LC-2 is that the covalent nature of the PROTAC may limit its potency as it cannot participate in catalytic rounds of degradation. Therefore, efforts to develop reversible PROTACs to target KRAS mutants are warranted. Despite its limitations, the discovery of LC-2 opens new opportunities for targeting KRAS mutants in cancer therapy.

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Declaration of Competing Interests:

The authors declare the following competing financial interest(s): C.M.C is founder, shareholder, and consultant to Arvinas, Inc., which supports research in his laboratory.

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