Chemical Proteomic Characterization of a Covalent KRASG12C Inhibitor

Aruna Wijeratne,^{†,§} Junpeng Xiao,[†] Christopher Reutter,[†] Kelly W. Furness,[†] Rebecca Leon,[†] Mohammad Zia-Ebrahimi,[†] Rachel N. Cavitt,[†] John M. Strelow,[†] Robert D. Van Horn,[‡] Sheng-Bin Peng,[‡] David A. Barda,[†] Thomas A. Engler,[†] and Michael J. Chalmers^{*,†,§}

[†]Discovery Chemistry Research and Technologies and [‡]Oncology Research, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285, United States

Supporting Information

ABSTRACT: The KRASG12C protein product is an attractive, yet challenging, target for small molecule inhibition. One option for therapeutic intervention is to design small molecule ligands capable of binding to and inactivating KRASG12C via formation of a covalent bond to the sulfhydryl group of cysteine 12. In order to better understand the cellular off-target interactions of **Compound 1**, a covalent KRASG12C inhibitor, we have completed a series of complementary chemical proteomics experiments in H358 cells. A new thiol reactive probe (TRP) was designed and used to construct a cellular target occupancy assay for KRASG12C. In addition, the thiol reactive probes allowed us to profile potential off-target interactions of **Compound 1** with over 3200 cysteine residues. In order to complement the TRP data we designed **Compound 2**, an alkyne containing version of **Compound 1**, to serve as bait in competitive chemical proteomics experiments. Herein, we describe and compare data from both the TRP and the click chemistry probe pull down experiments.

KEYWORDS: Chemical proteomics, mass spectrometry, covalent inhibitor, target identification, KRASG12C

In order to better understand the mechanism of action of small molecule drugs, it is important to identify the cellular protein targets of the ligand, as well as to measure the binding affinities of the ligand for those targets. Clearly this is a complex problem with no single experimental solution. However, mass spectrometry based chemical proteomic methods provide a consistently evolving set of tools to allow us to begin to elucidate such information.^{1–3}

ABPP methods employ probes designed to bind to a specific subset of proteins, such as enzymes, or to react with a specific amino acid, such as cysteine or lysine residues.^{4–6} For probe pull down experiments, a probe closely related to the structure of the test molecule is used as bait to enrich target proteins.⁷ During the purification steps of ABPP and probe pull down experiments, a significant number of nonspecific proteins will be isolated alongside probe labeled targets. Therefore, the observation of competition between probe and the unmodified test compound provides the critical evidence linking a test molecule to the target. Each of these two strategies is likely to identify unique and

common targets for a given ligand; therefore, a comprehensive chemical proteomics evaluation of a target molecule would ideally compare data obtained from multiple approaches.

Mutations in the KRAS oncogene are prevalent across a number of human cancers, and in certain diseases, mutant forms of the gene have been detected in over 90% of tumors.⁸ Within nonsmall cell lung carcinomas (NSCLCs), a gain of function mutation of glycine to cysteine at codon 12 accounts for ~40% of the mutant population.⁹ It was recently demonstrated how electrophile containing small molecule inhibitors were able to inactivate KRASG12C via formation of a covalent bond to the sulfhydryl group of cysteine 12.¹⁰ This exciting publication highlighted the ability of small molecule inhibitors to selectively

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target the oncogenic protein product (G12C) over the wild type KRAS. Following a mass spectrometry based screen of reactive fragments, a crystal structure was obtained, revealing fragments located in a new pocket, termed switch II (SWII). More advanced compounds, such as “Compound 12”, were then shown to exhibit a preference for the inactive GDP-loaded state of KRASG12C. Even though KRASG12C predominantly exists in cells in the GTP-loaded active state, it was shown that background rates of GTP hydrolysis do provide access to the inactive GDP bound state, therefore facilitating activity of “Compound 12” in cellular viability assays.¹¹ These data indicate that targeting the inactive GDP bound KRAS might provide a new opportunity for development of anti-KRASG12C drugs.^{10–13}

Building from the work described above,¹⁰ Patricelli and co-workers proceeded to discover ARS-853, an exciting new covalent small molecule KRASG12C inhibitor.¹¹ Making exquisite use of a complement of biochemical, structural biology, mass spectrometry, proteomics, and cellular methods, Patricelli and co-workers demonstrate binding of ARS-853 to the inactive GDP form of KRASG12C in the SWII pocket. ARS-853 was also demonstrated to have a biochemical rate of inactivation (k_{inact}/K_i) of $76 \text{ M}^{-1} \text{ s}^{-1}$ and a measured cellular IC_{50} of $1.6 \text{ } \mu\text{M}$ at 6 h.

In this work, we have synthesized **Compound (Cmpd) 1**, a covalent KRASG12C inhibitor described within a recent patent.¹⁴ In order to better understand the mechanism of action, we have completed a biochemical characterization of the k_{inact}/K_i value alongside cellular potency in a number of assays. In addition, we undertook a comprehensive chemical proteomics evaluation of the inhibitor in KRASG12C mutant expressing H358 cells. To this end, we designed a novel, isotope labeled, thiol reactive probe (TRP) and built a robust MS based target occupancy assay. In addition, the thiol probes were also used in a discovery experiment to identify off-target interactions. To complement the TRP target identification strategy, we synthesized an alkyne modified version of **Cmpd1** for use as bait in a competitive tandem mass tag click-chemistry pull down experiment. Here, we summarize the results from these techniques and compare the data and relative attributes of each approach. Following the publication of ARS-853,¹¹ we recognized a series of more advanced KRASG12C SWII inhibitors detailed within a subsequent patent publication.¹⁴ Following the protocols outlined in the patent, we synthesized **Cmpd1** (patent example 353), the structure of which is shown in Figure 1A. [Note that, following the patent disclosure of **Cmpd1** and the completion of our work described here, a single enantiomer of **Cmpd1** (ARS-1620) was published.]¹⁵

In order to measure the k_{inact}/K_i ratio for **Cmpd1** we performed a kinetic experiment in which we used mass spectrometry to measure the formation of the covalent adduct at Cys12 over time.¹⁶ Figure 1B shows the kinetic data obtained at different concentrations of **Cmpd1** (KRASG12C concentration was fixed at $2 \text{ } \mu\text{M}$). From these data we calculated a k_{inact}/K_i value of $501 \text{ M}^{-1} \text{ s}^{-1}$, a 6-fold increase when compared to ARS-853 (Figure 1C). To profile the cellular activity of **Cmpd1**, we performed cell viability assays in a number of KRASG12C cell lines (Table 1).

To develop a cellular target occupancy assay for KRASG12C; we designed a pair of thiol reactive probes (**Compounds 3 and 4**) as shown in Figure 2A. Inspired by others,^{11,17} we used iodoacetamide as the thiol reactive warhead and included a valine residue to serve as a light, or heavy, isotope label.¹⁷ The warhead and isotope label are connected via a PEG linker to a

Figure 1. (A) KRASG12C inhibitor (**1**) and alkyne chemical probe (**2**). (B) Data from a biochemical intact protein LC MS data assay to measure the fraction covalent adduct vs time. (C) k_{inact}/K_i values for **Cmpd1**.

Table 1. Measured Cell Potencies of Cmpd1

cell type	IC_{50} (μM)
H358	0.64
H23	0.70
H2030	0.16
MiaPaca-2	0.12

des-thio biotin tag that can be used as a handle for streptavidin pull down.¹¹ Unlike biotin, the des-thio biotin can be released from streptavidin with organic solvent and is therefore well suited to automated purification protocols. Because these probes are presumed to be noncell permeable, we add them after cell lysis and denaturation.

Therefore, we expect them to label all available cysteine residues. Figure 2B shows the mass spectrum obtained following the tryptic digest of a 1:1 mix of purified KRASG12C protein labeled with **Compounds 3 and 4**. The ions at m/z 778.466 and 781.473 are the $[\text{M} + 2\text{H}]^{2+}$ ions corresponding to the KRASG12C containing tryptic peptide, LVVVGAC*GVGK. As shown in Figure 2C, the pair of TRP probe labeled tryptic peptides coeluted at 36.8 min in our UHPLC Q-SIM method, and the AUC values were used to measure the H/L ratio (Supporting Information). In addition to the $[\text{M} + 2\text{H}]^{2+}$ ions, we also detected strong signals from the $[\text{M} + 3\text{H}]^{3+}$ ions (data not shown). To measure the target occupancy for **Cmpd1** and calculate an IC_{50} , we treated H358 cells with different concentrations of **Cmpd1** (DMSO, 0.08, 0.4, 2, and $10 \text{ } \mu\text{M}$) for 4 h. Each of these five treatments was paired with a matched set of DMSO control treated cells (Figure 2D). Compound treated cells were labeled with the heavy probe (**4**) and the control cells were labeled with the light probe (**3**). Lysates were then combined equally, and the probe labeled tryptic peptides were purified with automated streptavidin IP (Supporting Information). Following MS analysis, the AUC ratio of H/L for the KRASG12C tryptic peptide LVVVGAC*GVGK was used as an indicator of target occupancy as shown in Figure 2E. Cell treatments were performed in duplicate, and the sample was split across four IP tips. Half of these samples were used for the target occupancy experiment. Therefore, each data point shown in Figure 2E represents the average of four MS injections. The calculated IC_{50} of $1.8 \text{ } \mu\text{M}$ (after 4 h treatment) matched well with predicted values based on our measure biochemical k_{inact}/K_i value of $501 \text{ M}^{-1} \text{ s}^{-1}$.

Having demonstrated the utility of the thiol reactive probes for target engagement, we then employed them in a discovery proteomics experiment to search for additional covalent targets of **Cmpd1**. To achieve this, the samples described above (five

Figure 2. High-resolution MS KRASG12C target occupancy assay (UHPLC Q-SIM). (A) Thiol reactive probes (TRP). (B) Mass spectrum obtained from LC MS analysis of a trypsin digest of a 1:1 mixture of recombinant KRASG12C labeled with equal amounts of **Compounds 3** and **4**, respectively. (C) Extracted ion chromatograms obtained from the light and heavy labeled peptides shown in panel B. (D) Cellular TO assay workflow. (E) Concentration response curve showing cellular TO (H/L ratio) vs concentration of **Cmpd1**. A cellular IC_{50} of 1.8 μM was calculated from this data.

Figure 3. Discovery TRP experiments to identify covalent targets of **Cmpd1**. Following the scheme shown in Figure 2D, a series of five pairwise cellular experiments were prepared in biological duplicates (DMSO vs DMSO, 0.08, 0.4, 2, and 10 μM ; 4 h treatment). (A) Histogram of H/L ratios for two biological replicates of the DMSO vs DMSO samples. (B) Cross-plots showing log_2 H/L ratios for DMSO vs DMSO ($n = 3423$) and DMSO vs 2 μM ($n = 3301$) experiments (axis show biorep 1 vs biorep 2). (C) Full concentration response curves for six peptides showing dose-dependent reduction of log_2 H/L ratio upon compound treatment.

treatments, two cell replicates, two IPs) were profiled with extended data dependent nano LC MS/MS experiments (180 min gradients). Each sample was measured twice (40 LC MS/MS experiments in total), and the data were processed in MaxQuant¹⁸ to identify peptides and calculate the AUC ratios for each treatment (complete data table provided in Supporting Information). In total, 6293 peptides and 2917 proteins were identified. In all five treatments, there was good agreement

between the two biological replicates. For replicate one, AUC H/L ratios could be calculated for 3985 peptides. For replicate two, AUC H/L ratios were calculated for 3816 peptides. Figure 3A shows histograms of these data following Log_2 transformation of the H/L ratios (equal abundance of H and L = zero). Figure 3B shows a pair of cross-plots in which the log_2 H/L ratios for each peptide are plotted in the x-axis for replicate one and the y-axis for replicate two.

The upper cross plot shows the DMSO vs DMSO data and the lower cross plot shows data for DMSO vs 2 μ M **Cmpd1**. As expected, the KRASG12C tryptic peptide showed no competition in the DMSO vs DMSO experiment (0.16, 0.09), but was competed in the DMSO vs 2 μ M experiment (-0.98 , -1.24). Cross plots for all five treatments are provided in the [Supporting Information](#). In order to identify potential off targets, we filtered the data as described in the [Supporting Information](#). Briefly, we required that the peptide showed competition below -0.776 , -0.776 ($-0.776 = 3\sigma$ of the $\log_2$ H/L ratios) in one of the five treatments, and that the H/L ratio reduced in response to increasing dose of **Cmpd1**. Despite profiling over 3000 peptides, only six peptides showed any dose response with treatment of compound, as shown in [Figure 3C](#) (ordered with decreasing potency): KRAS (1.6 μ M), VAT1 (4.5 μ M and 4.5 μ M), HMOX2 (7.6 μ M), CRYZ (8.4 μ M), and RTN4 (>10 μ M). The KRASG12C tryptic peptide was shown to exhibit a dose-dependent reduction in H/L ratio, and the measured IC_{50} value (1.6 μ M) was consistent with the target occupancy data presented in [Figure 2](#) (1.8 μ M). In a similar thiol probe experiment,¹¹ Patricelli identified KRAS, FAM213A, and RTN4 as targets of ARS-853 (we did not identify any peptides from FAM213A in the TRP experiment for **Cmpd1**). Together, the results from these two TRP experiments suggest that KRASG12C inhibitors such as ARS-853 and **Cmpd1** do not covalently modify more than a handful of off-target proteins, even after several hours of cell treatment at high concentrations.

To complement the thiol probe study described above, we designed an alkyne probe (**Cmpd2**) to use as a bait compound in a click-chemistry pull down experiment ([Figures 4 and 5](#)).

Figure 4. Click chemistry target identification experiment with alkyne probe (**Cmpd2**). (A) Scheme highlighting progression from **Cmpd1** to **Cmpd2**. (B) Three biological replicates were prepared for each treatment and combined into a single TMT sample (DMSO vs 10 μ M **Cmpd1**). The sample was then separated into 24 $\times$ 1 min fractions with high pH HPLC. Each fraction was then analyzed twice by nano LC MS/MS. (C) Normalized KRASG12C TMT reporter ion intensities for LC MS/MS replicate 1.

Substitution at the 2-position of the quinazoline core of **Cmpd1** allowed for tethering of the alkynyl group while maintaining sufficient target binding, as anticipated by the SAR in the patent.¹⁷ The experimental design for the TMT click-chemistry pull down experiment is shown in [Figure 4B](#). H358 cells were first treated with DMSO ($n = 3$) or 10 μ M **Cmpd1** ($n = 3$) for 3 h, and then 2 μ M **Cmpd2** was added to all six treatments for an

additional 3 h. Cells were then lysed, clicked with azide-agarose resin, digested, labeled with TMT reagents, and combined into a single sample. To reduce the complexity of the sample prior to MS analysis, high pH HPLC was used to separate the TMT labeled sample into 12 fractions ([Supporting Information](#)). Each fraction was analyzed with a 60 min gradient low pH nano LC MS/MS experiment (Run 1). The low pH nano LC MS/MS experiment was then repeated (Run 2). Data from both Run 1 and Run 2 were searched with MaxQuant to identify proteins and calculate normalized reporter ion intensities for each of the six TMT samples. The measured TMT intensities for KRASG12C are shown in [Figure 4C](#); the **Cmpd1** treated samples ($n = 3$) were reduced when compared to the DMSO samples ($n = 3$).

To identify other off-target proteins, we imported the MS data into JMP 13.1 (SAS Institute Inc.). [Figure 5A](#) shows a histogram of $\log_2$ Cmpd/DMSO ratios for all proteins identified ($n = 2611$). The mean was 0.10 with a standard deviation of 0.25. To assess statistical significance of differences between DMSO and compound treatment for all proteins, we performed a least-squared means test of the data. For KRASG12C, the $\log_2$ **Cmpd1**/DMSO ratio was -1.8 and the Prob > F value was calculated to be 0.000018 (significantly lower than our typical cut off value of <0.01). Following a false discovery rate correction in JMP, the FDR adjusted p -value for KRAS remained <0.05 with a value of 0.022). [Figure 5B](#) shows a plot of the $\log_2$ **Cmpd1**/DMSO ratio against the unadjusted Prob > F value ($\log_{10}$ scale) for all 2611 proteins.

The lower left quadrant shows four proteins (FAM213A, KRAS, FABP5, and ALDH1A3) that were competed by the addition of **Cmpd1** (criteria are $\log_2$ Cmpd/DMSO ratio < 0.75 (3σ), Prob > F < 0.001 , FDR adjusted p -value < 0.05). Other proteins (colored orange in [Figure 5B](#); C4orf48, SELT, RECQL5, and TK2) were identified with $\log_2$ Cmpd/DMSO ratio $\leftarrow 0.75$ (3σ) and Prob > F < 0.01 ; however, the FDR adjusted p values were > 0.05 . Therefore, more evidence would be required before we would conclude that there was a valid interaction between these proteins and **Cmpd1**. Two proteins identified in the TRP experiment (VAT1 and RTN4) were also identified in the TMT click-chemistry pull down experiment and showed some evidence for competition ($\log_2$ Cmpd/DMSO ratios were -0.6 and -0.4 , respectively). However, neither reached statistical significance in the least squared mean test. A complete table showing the $\log_2$ **Cmpd1**/DMSO ratios, Prob > F values and FDR adjusted p -values for all 2611 proteins are provided in the [Supporting Information](#).

A summary of the results from the discovery TRP and the TMT click-chemistry pull down experiments are shown in [Table 2](#). As would be expected, all experiments provided robust data supporting cellular engagement of **Cmpd1** with its design target, KRASG12C. A number of additional off-targets were supported by data from TRP and TMT click-chemistry pull down. FAM213A showed strong evidence for binding in the click probe pull down experiment; however, no FAM213A peptides were identified in our TRP experiment. FABP5 showed strong evidence for binding in the click probe pull down experiment, and the TRP data did display dose response with treatment; however, the $\log_2$ H/L ratio for the 10 μ M dose did not exceed 3σ and therefore did not meet our "hit" criteria for calculation of IC_{50} . ALDH1A3 showed clear evidence for target engagement in the TMT click pull down experiment; however, the ALDH1A3 peptide identified in the TRP experiment did not show dose response. It is possible that **Cmpd1** modifies a different cysteine residue from the one that was identified in the TRP experiment.

Figure 5. Click chemistry TMT target identification data summary (H358 cells). (A) $\log_2$ H/L ratios for 2611 proteins identified in both LC MS/MS replicates. (B) Plot showing the $\log_2$ H/L ratio (normalized TMT reporter intensities) vs calculated Prob > F from a Standard Least Squares ANOVA. Proteins exhibiting statistically significant competition between alkyne probe 2 and the unmodified test compound, 1, are located in the lower left quadrant. No proteins were increased with compound treatment (lower right quadrant).

Table 2. H358 Proteomics Data Summary.^a

protein	Uniprot	thiol probe		mean $\log_2$ H/L	click probe pull down			thiol probe
		Cys	IC ₅₀ (μ M)		$\log_2$ H/L	Prob > F	FDR adj. <i>p</i> value	ARS-853 ^d
KRAS	P01116	12	1.8 ^b	0.2, 0.2, 0.0, -0.9, -3.9 ^b	-1.8	<0.0001	0.022	yes
		12	1.6 ^c	0.1, 0.3, -0.1, -1.1, n/d ^c				
FAM213A	Q9BRX8	n/d	n/c	n/d, n/d, n/d, n/d, n/d	-1.7	<0.0001	0.008	yes
FABP5	Q01469	67	n/c	-0.1, -0.2, -0.2, -0.2, -0.6	-1.3	<0.0001	0.032	no
ALDH1A3	P47895	52	n/c	0.1, 0.2, 0.1, 0.4, 0.3	-1.1	<0.0001	0.049	no
VAT1	Q99536	86	4.5	0.0, 0.0, -0.4, -1.0, -1.1	-0.6	0.1326	0.997	no
		50	4.5	0.0, 0.1, -0.4, -0.9, -1.0				
RTN4	Q9NQC3	1101	14.6	0.1, 0.0, 0.0, -0.3, -1.1	-0.4	0.0639	0.999	yes
HMOX2	P30519	282	7.6	0.1, 0.1, 0.0, -0.2, -1.2	-0.3	0.2014	0.999	no
CRYZ	Q08257	45	8.4	-0.2, -0.2, -0.2, -0.3, -0.9	0.15	0.255	0.999	no

^aFrom left to right: protein name; Uniprot ID number; Cys residue identified in TRP; IC₅₀ calculated from TRP (4 h treatment); $\log_2$ H/L ratios for the five cell treatments shown in Figure 3 (DMSO, 0.08, 0.4, 2, 10 μ M); $\log_2$ H/L ratio from the TMT click probe pull down experiment shown in Figures 4 and 5; Prob > F value for least squared means test; FDR adjusted *p*-value; target of ARS-853 previously identified by Patricelli et al. n/d = no data. n/c = not calculated. ^bData from targeted ABPP TO assay (Figure 2). ^cData from discovery ABPP (Figure 3). ^dIdentified as a target of ARS-853 in ref 11.

RTN4, HMOX2, and CRYZ showed dose response in the TRP data but were not identified as a targets in the TMT click-chemistry pull down experiment. It is possible that the addition of the alkyne to the compound reduced, or eliminated, binding to these proteins. Our data highlight the complementary nature of chemical proteomics methods and support our comprehensive approach to the discovery of cellular targets of small molecules inhibitors. Each technique employed here offers advantages and has drawbacks. For example, the TRP experiment does not require any additional probe design and is quick to implement for a new test compound; however, TRP offers no enrichment of low abundance targets and requires detection of a single tryptic peptide for identification and quantification. It may therefore be expected to observe some number of false negatives in TRP experiments. The TMT click-chemistry pull down experiment allows for enrichment of targets and multiprotein identification and quantification. However, significant effort is required to design and characterize a suitable probe, and there is always the possibility of missing targets that may bind test compound, but not the probe; again leading to some number of false negatives.

In conclusion, our combined chemical proteomics workflow has provided a cellular target occupancy assay for KRASG12C and generated experimental evidence for the covalent binding of

Cmpd1 with KRASG12C, FAM213A, FABP5, ALDH1A3, VAT1, and RTN4. Having demonstrated modification of these targets, it is tempting to consider what the potential pharmacological or toxicological effects may be. Although RNT4 has been identified as a potential target for colorectal cancer,¹⁹ we do not observe any activity in cell viability assays with **Cmpd 1** in non-KRASG12C cell lines (data not shown). Therefore, we consider that the modification of these off targets are likely unrelated to our desired pharmacology. The methods described here are broadly applicable to other covalent small molecule inhibitor projects, and are expected to provide important information for future efforts. It is important to acknowledge that the protocols described here focus on covalent target discovery. Future effort will therefore focus on the development and application of additional chemical proteomics solutions designed to identify reversible off-target interactions.

EXPERIMENTAL PROCEDURES

Compound Synthesis. Compound 1 was synthesized following published methods.¹⁷ Synthetic methods and supporting analytical data Compounds 2–4 are provided in the Supporting Information. Complete methods for the TO assay, the discovery TRP work, and the click-chemistry pull down experiments are provided in the Supporting Information.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmmedchemlett.8b00110.

Comprehensive methods and MS data tables (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: chalmers_michael@lilly.com.

ORCID

Michael J. Chalmers: 0000-0002-2139-6089

Present Address

[§]Department of Biochemistry & Molecular Biology, Proteomics Core, Indiana University School of Medicine, Indianapolis, Indiana 46202, United States.

Author Contributions

The manuscript was written through contributions of all authors. Experimental data was generated and analyzed by A.W., J.X., C.R., K.F., M.Z., R.C., J.S., R.V.H., and M.C. Probes were designed by K.F., T.E., D.B., M.Z., and M.C. Studies were designed by A.W., J.X., K.F., R.L., J.S., S.P., D.B., T.E., and M.C. All authors contributed to writing of the manuscript.

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Notes

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■ ABBREVIATIONS

TMT, tandem mass tag; ABPP, activity based proteomics profiling; TRP, thiol reactive probe

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