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Deconstructing Lipid Kinase Inhibitors by Chemical Proteomics

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Abstract

Diacylglycerol kinases (DGKs) regulate lipid metabolism and cell signaling through ATP-dependent phosphorylation of diacylglycerol to biosynthesize phosphatidic acid. Selective chemical probes for studying DGKs are currently lacking and are needed to annotate isoform-specific functions of these elusive lipid kinases. Previously, we explored fragment-based approaches to discover a core fragment of DGK- α (DGK α) inhibitors responsible for selective binding to the DGK α active site. Here, we utilize quantitative chemical proteomics to deconstruct widely used DGK α inhibitors to identify structural regions mediating off-target activity. We tested the activity of a fragment (RLM001) derived from a nucleotide-like region found in the DGK α inhibitors R59022 and ritanserin and discovered that RLM001 mimics ATP in its ability to broadly compete at ATP-binding sites of DGK α as well as >60 native ATP-binding proteins (kinases and ATPases) detected in cell proteomes. Equipotent inhibition of activity-based probe labeling by RLM001 supports a contiguous ligand-binding site composed of C1, DAGKc, and DAGKa domains in the DGK α active site. Given the lack of available crystal structures of DGKs, our studies highlight the utility of chemical proteomics in revealing active-site features of lipid kinases to enable development of inhibitors with enhanced selectivity against the human proteome.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.7b00962. Experimental methods and supplementary figures (PDF) Table S1 (XLSX)

Diacylglycerol kinases (DGKs) are members of the lipid kinase superfamily that catalyze phosphorylation of diacylglycerol (DAG) to generate phosphatidic acid^{1–3} [PA (Figure S1A)]. Both DAG and PA serve as potent lipid messengers to shape cellular responses by altering the subcellular localization, activation, and function of essential receptor proteins (ranging from enzymes to transcription factors).^{4,5} DAG and PA also serve as building blocks for phospholipid and triglyceride biosynthesis and are integral to membrane architecture and bioenergetics.¹ To date, 10 mammalian DGKs have been identified, and comparative analysis of primary sequences has classified individual isoforms into five principal subtypes¹ [types 1–5 (Figure S1B)]. Mammalian DGKs are composed of at least two cysteine-rich zinc finger-like motifs analogous to C1 domains found in protein kinase C⁴ (PKC) and a C-terminal catalytic domain containing both a lipid kinase (DAGKc) and an accessory (DAGKa) subdomain.⁶ Individual isoforms are differentiated on the basis of protein regions with homology to domains known to mediate lipid, protein, and other small molecule interactions that are thought to control when and where DGKs are active.¹ Thus, DGKs hold enormous potential as therapeutic targets because of their fundamental role in sculpting the lipidome to support metabolic, structural, and signaling demands of cells but currently lack selective chemical probes for exploiting their isoform-specific biology.⁷

Recent studies have identified diacylglycerol kinase- α (DGK α) as a promising target for cancer immunotherapy because of its critical role in regulating lipid signaling necessary for proper T cell activation.^{6,8,9} T cell receptor signaling is mediated by secondary messengers, including diacylglycerols (DAGs) that act as ligands to alter the subcellular localization and activation of key kinase proteins (e.g., Ras/Erk) that are essential for T cell activation.¹⁰ DGK α negatively regulates TCR signaling by phosphorylating DAG to terminate its signaling activity¹¹ (Figure S1A). Excessive DGK α activity (and thus attenuated DAG signaling) has been linked to defective T cell function. In the clinic, tumor-infiltrating lymphocytes (TILs) isolated from renal carcinoma patients showed an increased level of expression of DGK α , which correlated with impaired cytotoxic responses that could be reversed with nonselective DGK α inhibitors.¹² Finally, DGK α inactivation in chimeric antigen receptor (CAR)-modified T cells (T cells genetically modified for tumor antigen specificity¹³) enhances immune responses against tumors.¹⁴ Thus, development of highly selective DGK α inhibitors is a promising therapeutic strategy for reversing immunosuppressive metabolic pathways operating in the tumor microenvironment. The challenge with developing DGK α -selective inhibitors is this lipid kinase along with >500 other mammalian kinases¹⁵ utilize ATP as a common substrate and targeting the canonical ATP-binding pocket will likely result in substantial off-target activity.

Previously, we initiated efforts to address the challenge of developing DGK α -selective inhibitors using chemical proteomics¹⁶ and quantitative mass spectrometry.^{17–19} Specifically, we utilized ATP acyl phosphate activity-based probes^{20,21} (Figure 1A) to discover ligand-binding sites mediating substrate and inhibitor recognition of DGK α along with representative members from all five DGK subtypes.²² From these studies, we identified DAGKc/DAGKa as the primary ATP-binding site of DGK α and the atypical C1 domain as a novel inhibitor-binding site of the dual DGK α /FER inhibitor ritanserin. We also discovered the DGK α lipid kinase inhibitory activity of ritanserin could be recapitulated by a fragment [RF001 (Figure 1B)] derived from a hydrophobic region of ritanserin with enhanced selectivity against protein kinases compared with that of the parent molecule.²²

Our approach was necessary because crystal structures of mammalian DGKs are currently not available. Compared with conventional substrate assays using purified protein, our chemical proteomics strategy enabled rapid evaluation of compound potency against DGKs and selectivity against other kinase activities detected in the cell proteome. Notably, we used chemical proteomics to identify the site of binding and quantify inhibition at respective binding sites for DGK inhibitors, which is a challenging task using traditional profiling methods. Finally, chemical proteomic profiling of DGKs in cell proteomes permits analysis in more native environments to preserve functional conformations of lipid kinases.

However, the structural region contributing to FER protein kinase off-target activity of ritanserin is unknown and critical for enabling fragment-based design of DGK α -selective inhibitors. In this report, our studies began with examination of chemical structures of commonly used DGK α inhibitors to identify a thiazolopyrimidinone region (Figure S2) common in ritanserin and R59022.^{23–25} The resemblance of this heterocycle to the adenine portion of ATP led us to hypothesize its role in mediating protein kinase off-target activity observed with ritanserin.²² We tested a fragment [designated as RLM001 (Figure 1B)] derived from this region for both DGK α and general kinase inhibitory activity using competitive gel-based chemical proteomics (Figure 2). First, we overexpressed recombinant DGK α in HEK293T cells, validated protein expression by Western blot (Figure S3A), and confirmed recombinant DGK α activity in soluble proteomes using our previously described DAG phosphorylation substrate assay.²² We observed significantly higher DAG phosphorylation activity in DGK α -overexpressed versus mock transfected soluble proteomes (Figure S3B). The specificity of recombinant DGK α activity was confirmed by demonstrating blockade of catalytic activity with both ritanserin and R59022 but not the negative control compound ketanserin^{22,24} (Figure S3B).

Next, we used ATP acyl phosphates as activity-based probes to evaluate the activity of RLM001 against recombinant DGK α . ATP acyl phosphate probes enable global profiling of kinase activities by covalent attachment of reporter tags [desthiobiotin (Figure 1A)] to conserved lysine residues in the ATP-binding site,^{20,21} and we recently adapted this probe for chemical proteomic profiling of DGKs.²² Using conditions optimized previously for fragment screening,²² we tested the potency and selectivity of RLM001 directly in recombinant DGK α -HEK293T soluble proteomes by chemical proteomics (Figure 2A). In brief, DGK α -HEK293T soluble proteomes were pretreated with varying concentrations of RLM001 (0.15–10 mM) followed by reaction with the ATP acyl phosphate probe,

desthiobiotin-modified proteins separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane, and probe-modified proteins detected using a fluorescently labeled streptavidin (Figure 2B).

Pretreatment with RLM001 resulted in concentration-dependent blockade of DGK α probe labeling as measured by the decreasing magnitudes of the fluorescent protein signals [IC_{50} = 4 mM, 95% confidence interval of 2–11 mM (Figure 2C)]. We also confirmed that potency values determined by chemical proteomics matched those measured using conventional DAG phosphorylation substrate assays [IC_{50} = 3 mM, 95% confidence interval of 1–19 mM (Figure 2D)]. These results support a role for the thiazolopyrimidinone group in mediating inhibitory activity of ritanserlin and R59022 against DGK α (Figure S3B). Surprisingly, we also observed global decreases in the magnitudes of fluorescent protein signals in HEK293T soluble proteomes with RLM001 treatments, especially at the higher concentrations (Figure 2B). Our results suggest RLM001 is broadly competing at ATP-binding sites of kinases as well as other ATP-binding proteins detected in cell proteomes. To test this concept, we directly compared the selectivity of RLM001 with free ATP to evaluate whether the former fragment mimics the latter kinase substrate in binding activity. The inhibition profiles of RLM001 (5 mM) and free ATP (1 mM) were indistinguishable with the exception of a handful of fluorescent protein bands that showed mild differences in competition (Figure 2B). We tested the selectivity of RLM001 at concentrations substantially higher than those of free ATP because low-molecular weight fragments such as RLM001 (196.18 Da) typically exhibit binding affinities in the millimolar range.²⁶

Next, we implemented a liquid chromatography–mass spectrometry (LC–MS) quantitative chemical proteomic assay to discover RLM001-binding site(s) of DGK α ²² (Figure S4). For these LC–MS studies, quantitation was enabled by stable isotope labeling with amino acids in cell culture [SILAC²⁷ (Figure S4)]. In brief, recombinant DGK α was overexpressed in isotopically light and heavy amino acid-labeled HEK293T cells. Light and heavy DGK α -HEK293T lysates were treated differentially with dimethyl sulfoxide (DMSO) vehicle and RLM001 (10 mM), respectively, prior to addition of ATP acyl phosphate to label active-site lysines. After probe labeling, light and heavy proteomes were combined and digested with trypsin, and desthiobiotin-modified peptides were enriched by avidin affinity chromatography and analyzed by LC–MS/MS to identify and quantify isotopically labeled active-site peptides from DGK α as previously described²² and depicted in Figure S4.

Probe-modified peptides showing a high level of competition, as judged by SILAC ratios (SR), were identified as RLM001-binding sites in DMSO/RLM001 comparisons [SR > 5 (Figure 3A and Table S1)]. We used these criteria to discover that all three probe-binding sites previously identified in DGK α active sites²² were highly sensitive to RLM001 competition at the highest concentration tested (10 mM): C1 (K237; SR > 20), DAGKc (K377; SR > 20), and DAGKa [K539; SR > 20 (Figure S5 and Table S1)]. We compared inhibition profiles of RLM001 directly with those of free ATP to determine whether these compounds bind at the same sites. Both RLM001 and ATP showed near-complete blockade of probe labeling at the primary ATP-binding sites (DAGKc/DAGKa) of DGK α (Figure S5). In contrast, RLM001 (SR > 20) strongly competed with the C1-binding site but ATP did not [SR ~ 2 (Figure S5)].

We also performed a RLM001 dose–response study to determine whether RLM001 inhibits all three DGK α active-site peptides with equal affinity. These studies would allow us to determine whether C1, DAGKc, and DAGKa domains form a contiguous binding site for RLM001 or whether distinct binding sites exist. As shown in Figure 3B, pretreatment with RLM001 resulted in the equipotent blockade of all three DGK α active-site peptides [$IC_{50} \sim 1\text{--}3\text{ mM}$ across C1, DAGKc, and DAGKa sites (Figure 3B and Table S1)]. Collectively, our results provide the first evidence that C1, DAGKc, and DAGKa form a connected ligand-binding site that mediates interactions with the thiazolopyrimidinone region of ritanserin.

Our gel-based findings provided initial evidence that RLM001 is broadly competing at ATP-binding sites across the kinome, but target identification and site of binding information are needed to validate this hypothesis. We used quantitative chemical proteomics (Figure S4) to demonstrate that RLM001 competes at ATP-binding sites of ~ 60 native kinases quantified in HEK293T soluble proteomes (Figure 3A). All probe-modified peptides from native kinases reported in Figure 3 and Table S1 were manually inspected for quality control as previously described.²² Native kinase targets of RLM001 were identified by active-site peptides showing SILAC ratios of ~ 5 .

We discovered that inhibition profiles of kinase active-site peptides from RLM001- versus ATP-treated samples were largely indistinguishable (Figure 3A), matching the results from our gel-based chemical proteomics (Figure 2B). Closer inspection of individual active-site peptides from several example protein kinases, including BRAF and MAP2K1/2 (also known as MEK1/2), which are critical kinase regulators of oncogenic signaling,²⁸ clearly demonstrates the ability of RLM001 to compete efficiently at respective ATP-binding sites [for BRAF and K578, $SR > 20$ (Figure 3C); for MAP2K1, K192/MAP2K2, and K196, $SR > 20$ (Figure S6)]. Globally, this trend is recapitulated across $\sim 90\%$ of kinases detected in HEK293T soluble proteomes in our LC–MS analyses (Figure 3A and Table S1).

We also performed dose–response studies to determine whether specific classes of protein kinases show differences in sensitivity to RLM001 competition at respective active sites. For these studies, we chose to evaluate a representative member from the TKL (BRAF), CMGC (CDK5), CAMK (CHK2), TK (CSK), AGC (KS6A1), and STE (M3K2) protein kinase classes.¹⁵ We found that all protein kinases tested showed comparable sensitivity to RLM001 competition at respective ATP-binding sites with potency (IC_{50}) values ranging from 1 to 3 mM (Figure 3D and Table S1). We also tested whether RLM001 can compete at active sites of ATPases²⁹ [heat shock proteins (HSPs) (Figure 3C)]. We found that both ATP and RLM001 can compete at ATP-binding pockets of HSP90 (HSP90 α , K112/HSP90 β , K107; for ATP, $SR > 20$; for RLM001, $SR > 20$) and HSP70 proteins [HSP70-1A, K56/HSP70-1B, K56; for ATP, $SR = 5.2$; for RLM001, $SR = 19.1$ (Figure 3C and Table S1)]. We performed dose–response studies to determine whether HSPs show differences in sensitivity to RLM001 treatments and found that potency values were comparable between HSP90 ($IC_{50} = 2.8\text{ mM}$, 95% confidence interval of 1.9–4.1 mM) and HSP70 [$IC_{50} = 1.0\text{ mM}$, 95% confidence interval of 0.4–2.5 mM (Figure 3E and Table S1)]. Collectively, the comparable potency of RLM001 against kinases and ATPases supports this compound as a general ligand for ATP-binding pockets.

Interestingly, not all protein kinase active sites show equivalent sensitivity to RLM001 and ATP competition. For example, we discovered that RLM001 treatments do not completely block probe labeling of MARK1/2 ATP-binding sites [MARK1, K89/MARK2, K82; SR = 4.1 (Figure S6)]. The functional implications of this differential sensitivity are not clear but could provide interesting insights into MARK1/2 function, which are implicated in phosphorylating microtubule-associated proteins, including Tau.³⁰ In summary, our results demonstrate that the RLM001 fragment broadly competes at ATP-binding sites, supporting our hypothesis that the RLM001 region of ritanserin contributes to off-target activity.

DGKs are responsible for balancing intracellular levels of DAG and PA lipids at the interface of membrane architecture, bioenergetics, and cellular signaling.^{1–3} How these complex tasks are accomplished through the metabolic activity of 10 mammalian DGK isoforms is currently unknown and being explored. Development of isoform-selective inhibitors would greatly advance our ability to probe the function of these lipid kinases *in vivo*. Here, we have taken important steps to address this challenge by deconstructing known DGK inhibitors to identify structural regions contributing to on- versus off-target activity. We hypothesized that the thiazolopyrimidinone region of widely used DGK α inhibitors contributes to protein kinase off-target activity based on the structural resemblance to the nucleotide portion of ATP. We tested our hypothesis by using quantitative chemical proteomics to demonstrate that a fragment (RLM001) derived from both ritanserin and R59022 mimics ATP in its ability to bind active sites of DGK α as well as >60 ATP-binding proteins, including protein kinases and ATPases detected in cell proteomes. Our studies also provide the first evidence that the C1, DAGKc, and DAGKa domains of DGK α form a contiguous binding site for ligands such as RLM001. Given the emerging role of DGK α for immuno-oncology,^{6,8,9,12,14} we believe our findings will guide future development of DGK α inhibitors with enhanced selectivity against the human proteome to mitigate potential toxicity associated with off-target activity.

While RLM001 is likely a poor candidate for developing selective lipid kinase inhibitors, this fragment has attractive features as a starting point for developing an activity-based probe, including broad reactivity at ATP-binding sites of kinases and other ATP-binding proteins (e.g., ATPases). We envision the incorporation of electrophilic groups into the RLM001 scaffold to mediate covalent reaction with nucleophilic residues (e.g., cysteine and/or lysines) in ATP-binding pockets for live cell activity-based profiling studies. Inclusion of an alkyne handle will facilitate downstream detection of direct protein targets of RLM001 activity-based probes. Furthermore, conversion of RLM001 from a reversible fragment to a covalent activity-based probe should help mitigate the poor potency observed with the parent molecule by taking advantage of the non-equilibrium binding mechanism of covalent compounds to lower the required concentration needed for activity.³¹

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Evaluating the activity of DGK α inhibitor fragments using kinase activity-based probes. (A) Mechanism for ATP acyl phosphate probe reaction. The nucleophilic ϵ -amine group of lysine side chains attacks the acyl phosphate, resulting in covalent modification of kinase active sites with a desthiobiotin tag for downstream detection via gel- or mass spectrometry-based readouts. (B) Ritanserin is a dual DGK α /FER kinase inhibitor that is deconstructed to evaluate the resulting fragments for lipid vs protein kinase activity. RF001 was previously shown to mediate selective inactivation of the lipid kinase DGK α .²² This study will test whether RLM001 mediates general off-target activity of ritanserin against ATP-binding sites.

Figure 2.

Gel-based chemical proteomics for evaluating the potency and selectivity of RLM001. (A) Schematic of competitive gel-based chemical proteomics using ATP acyl phosphates to screen fragments for kinase binding activity. (B) Gel-based ATP acyl phosphate assay used to determine *in vitro* IC₅₀ values for DGKα inhibition by RLM001 (10, 5, 1, 0.25, and 0.15 mM). Western blot analysis (anti-FLAG, 0.8 mg/mL) confirmed equivalent recombinant DGKα expression across treatment conditions. (C) Dose–response curve of the gel-based ATP acyl phosphate assay to determine RLM001 potency (IC₅₀). Data are means ± the standard error of the mean for five biological replicates; 95% confidence intervals for IC₅₀ values of 2–11 mM. (D) Dose–response curve of the DAG phosphorylation substrate assay to determine RLM001 potency (IC₅₀). Data are means ± the standard error of the mean for two biological replicates; 95% confidence intervals for IC₅₀ values of 1–19 mM.

Figure 3.

Identification of RLM001-binding sites by quantitative chemical proteomics. (A) Heat map showing the potency and selectivity of ATP (1 mM) and RLM001 (10 mM) against recombinant DGK α and native kinases detected in HEK293T proteomes. Kinases containing multiple probe-binding sites are differentiated by unique lysine-modified positions shown in parentheses. Peptide ratios for the heat map shown are listed in Table S1. (B) Dose-response curves to determine the potency (IC_{50}) of RLM001 at DGK α active-site peptides: C1, IC_{50} = 2.6 mM, 95% confidence interval (CI) of 0.5–13.7 mM; DAGKc, IC_{50} = 1.1 mM, 95% CI of 0.4–2.6 mM; DAGK α , IC_{50} = 1.9 mM, 95% CI of 0.9–3.8 mM. (C) MS2 spectra (left) of probe-modified peptides corresponding to BRAF, HSP90 α/β , and HSP70-1A/B. Major b- and y-ions produced by fragmentation of the precursor peptide ion (MS1) are indicated on the spectrum in red. Probe-modified lysines are indicated in red on the peptide sequence. MS1-extracted ion chromatograms (right) of probe-modified peptides with corresponding SILAC ratios quantifying vehicle (light) or compound treatment (heavy). MS1 peptides for BRAF, HSP90 α/β , and HSP70-1A/B are all potently inhibited by both ATP (1 mM) and RLM001 (10 mM) as defined by SR > 5. (D) Dose-response curves to determine the potency of RLM001 against members of different protein classes: BRAF, IC_{50} = 0.9 mM, 95% CI of 0.2–3.8 mM; CDK5, IC_{50} = 2.1 mM, 95% CI of 1.1–4.2 mM; CHK2, IC_{50} = 1.2 mM, 95% CI of 0.7–2.2 mM; CSK, IC_{50} = 1.6 mM, 95% CI of 0.8–3.3 mM; KS6A1, IC_{50} = 0.8 mM, 95% CI of 0.4–1.4 mM; M3K2, IC_{50} = 3.3 mM, 95% CI of 0.8–14 mM. (E) Dose-response curves to determine the potency of RLM001 against ATPases: HSP90 α/β , IC_{50} = 2.8 mM, 95% CI of 1.9–4.1 mM; HSP70-1A/B, IC_{50} = 1.0 mM, 95% CI of 0.4–2.5 mM.