SUPPORTING INFORMATION

Chemoproteomic profiling of kinases in live cells using electrophilic sulfonyl triazole probes

Tao Huang¹, Seyyedmohsen Hosseinibarkooie⁵, Adam L. Borne², Mitchell E. Granade², Jeffrey W. Brulet¹, Thurl E. Harris², Heather A. Ferris⁵, and Ku-Lung Hsu*^{1,2,3,4}

¹Department of Chemistry, University of Virginia, Charlottesville, Virginia 22904, United States

²Department of Pharmacology, University of Virginia School of Medicine, Charlottesville, Virginia 22908, United States

³University of Virginia Cancer Center, University of Virginia, Charlottesville, VA 22903, USA

⁴Department of Molecular Physiology and Biological Physics, University of Virginia, Charlottesville, Virginia 22908, United States

⁵Department of Medicine, University of Virginia School of Medicine, Charlottesville, Virginia 22903, United States

*Author to whom correspondence should be addressed: K.-L.H. (kenhsu@virginia.edu)

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1. SUPPORTING FIGURES



Figure S1. AG modifications tune reactivity of RF001-SuTEx probes. Gel-based chemical proteomic evaluation of HEK293T cell proteomes treated with RF001-SuTEx probes (100 μ M, 1 hr, 37 °C) shows that AG modifications affects proteome reactivity of probes. All data shown are representative of 2 experiments (n = 2 biologically independent experiments)



Figure S2. RF001 binding element directs molecular recognition of SuTEx probes. The DGK α binding element RF001 directs molecular recognition of RF001-SuTEx probes as evidenced by concentration-dependent labeling of recombinant rat DGK α (rDGK α) overexpressed in HEK293T cell proteomes (10-100 μ M TH211, 1 hr, 37 °C). Mock transfected proteomes were included to aid in identification of recombinant HEK293T cell proteomes in gelbased chemical proteomic studies. All data shown are representative of 2 experiments (n = 2 biologically independent experiments)



Figure S3. Quantitative chemical proteomic evaluation of recombinant human DGK α -HEK293T soluble proteomes. Probe-modified tyrosine sites identified and shared between TH211, TH214, and TH216 probes. Tyrosine sites shown are specifically enriched (light – probe / heavy – vehicle SILAC ratios >5) and meet our peptide quality control criteria as described in Supporting Methods. A list of all probe-modified tyrosines sites can be found in Table S1. RVH, recoverin homology domain; EF, EF Hands motif; C1, atypical/typical C1 domain.



Figure S4. TH211 is active in live cells. Jurkat cells were treated with TH211 at varying concentrations (10-100 μ M, 2 hrs, 37 °C) followed cell lysis and gel-based chemical proteomic analysis of soluble and membrane proteomes. All data shown are representative of 2 experiments (n = 2 biologically independent experiments).





(A and B) The catalytic activity of recombinant human DGK α and Y240F mutant were evaluated by a liposomal substrate assay against C18:1_C18:1 DAG substrate (n = 3 biological samples). The Y240F mutant was significantly catalytically impaired compared with wild-type protein. Data shown represent mean ± s.d. **P < 0.01 for Y240F versus wild-type DGK α . (C) Protein sequence alignment shows DGKA Y240 is evolutionary conserved among different species.



Reactome pathways enrichment analysis

Figure S6. TH211-modified protein kinases are enriched for signal transduction pathways. Top 20 Reactome pathways from enrichment analysis of probe-modified protein kinases identified in TH211-treated Jurkat cells (50 μ M probes, 2 hrs, 37 °C). Kinases used for analyses showed specific enrichment based on SILAC ratios from light – TH211 / heavy – vehicle comparisons. Kinases included in the sample space were determined by using reviewed human proteins classified under the kinase keyword KW-0418 in UniProt (<u>https://www.uniprot.org/</u>).



Reactome enrichment analysis (non-protein kinases)

Figure S7. Reactome pathways enrichment analysis of TH211-modified non-protein kinases. Enrichment analyses was performed on probe-modified non-protein kinases identified in TH211-treated Jurkat cells (50 μ M probes, 2 hrs, 37 °C). Kinases used for analyses showed specific enrichment based on SILAC ratios from light – TH211 / heavy – vehicle comparisons. Kinases used for the sample space were determined by using reviewed human proteins from the kinase keyword KW-0418 in UniProt (https://www.uniprot.org/).



Figure S8. Functional analysis of probe-modified sites in the C2 in PMA-mediated downregulation of PKC- α . HEK293T cells expressing wild-type or mutant PKC- α (PRKCA) were treated with PMA (500 nM, 6 hrs) followed by western blot analysis of protein signals (anti-Flag) to determine PMA-mediated downregulation through protein degradation. Actin signals were used as a loading control for analyses.

PRKCA	Y195		K209		K232
Drosophila	DPNGLSDPYVKVKLIPDDK	Drosophila	DQSKKKTRTIKACLN	Drosophila	ETLTYDLKPEDKDRRI
C.elegans	DPNGLSDPYVKCKLIPEDS	C.elegans	CKSKQKTKTLRATLN	C.elegans	ETFTYKLLPGDKDRRL;
Mouse	DPNGLSDPYVKLKLIPDPK	Mouse	NESKQKTKTIRSTLN	Mouse	ESFTFKLKPSDKDRRL
Human	DPNGLSDPYVKLKLIPDPK	Human	NESKQKTKTIRSTLN	Human	ESFTFKLKPSDKDRRL
Monkey	DPNGLSDPYVKLKLIPDPK	Monkey	NESKQKTKTIRSTLN	Monkey	ESFTFKLKPSDKDRRL
Zebrafish	DPNGLSDPYVKLKLIPDPK	Zebrafish	NETKQKTKTIRSSLN	Zebrafish	ETFTFKLKPADKDRRL
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Figure S9. TH211-modified sites in PKC- α (PRKCA) C2 domain are evolutionarily conserved.

2. CHEMICAL SYNTHESIS

All chemical reagents and solvents were obtained from commercial suppliers such as Sigma Aldrich, Acros, Alfa Aesar, Combi-Blocks, and used as supplied without further purification unless stated otherwise. Analytical thin layer chromatography (TLC) was performed on Merck Silica gel 60 F254 plates (0.25 mm). Preparative TLC was conducted on Analtech Silica gel GF UV254 (1000 micron). Flash column chromatography was accomplished with Silica Gel 60 (230-400 mesh) purchased from Fisher Scientific. Detection was accomplished by using UV-light (254 nm) and/or TLC staining reagent such as phosphomolybdic acid (PMA). Nuclear magnetic resonance (NMR) spectra (¹H and ¹³C) were recorded on a Varian spectrometer at 600 MHz at room temperature. Chemical shifts were provided in parts per million (ppm) with coupling constants in Hz. ¹H and ¹³C spectra were calibrated in relation to deuterated solvents, namely CDCl3 (7.26 ppm for ¹H and 77.16 ppm for ¹³C) and CD₃COCD₃ (2.05 ppm for ¹H, 29.84 and 206.26 ppm for ¹³C). Splitting patterns for apparent multiplets were indicated as s (singlet), d (doublet), t (triplet), g (quartet), m (multiplet), br (broadened) as well as combinations of them. High resolution mass spectrometry was obtained with an Agilent 6545B LC/Q-TOF (Agilent Technologies, Santa Clara, CA, USA). Purity of all final products was greater than 97% as determined by analytical high performance liquid chromatography (HPLC) with a Shimadzu 1100 Series spectrometer detector.



tert-Butyl 4-(bis(4-fluorophenyl)(hydroxy)methyl)piperidine-1-carboxylate (1)

The synthetic procedure was performed following previously described methods¹. To a cooled solution of 1-Boc-isonipecotic acid ethyl ester (7.7 g, 30 mmol) in anhydrous THF (60 mL) at 0 °C was added 4-fluorophenyl magnesium bromide (2 M in diethyl ether, 36 mL, 72 mmol) over 20 min. The reaction mixture was warmed to room temperature and stirred overnight (14 hr) and then quenched with aqueous NH₄Cl solution. The aqueous layer was extracted with EtOAc 3 times and the combined organic layer was washed with brine, dried over MgSO₄ and concentrated on rotary evaporator. The crude material was dissolved in hot EtOAc/Hexane (1:3) and then cooled to 4 °C, and the precipitate was collected to yield compound **1** (10.5 g, 87%) as white solid. ¹H NMR (600 MHz, CDCl₃) δ 7.46 – 7.34 (m, 4H), 6.95 (t, *J* = 8.7 Hz, 4H), 4.10 (d, *J* = 13.2 Hz, 2H), 2.66 (t, *J* = 12.8 Hz, 2H), 2.54 (broad, 1H), 2.45 (tt, *J* = 11.9, 3.1 Hz, 1H), 1.43 (m, 2H), 1.39 (s, 9H), 1.27 (qd, *J* = 12.6, 4.4 Hz, 2H). ¹³C NMR (150 MHz, CDCl₃) δ 162.30, 160.67, 154.63, 141.36, 141.34, 127.58, 127.52, 115.05, 114.91, 79.45, 79.00, 44.47, 43.93, 28.37, 26.40.



4-(Bis(4-fluorophenyl)methylene)piperidine (2, RF001)

A mixture of **1** (2.02 g, 5 mmol) and trifluoroacetic acid (10 mL) in $CH_2Cl_2(10 \text{ mL})$ was stirred at room temperature for overnight (14 hr) and then concentrated. The residue was diluted with NaOH (1 N) and extracted with CH_2Cl_2 several times. The combined organic layers were washed with brine, dried over MgSO4 and concentrated in vacuum to afford compound **2** (1.34 g, 94%) as a white solid. ¹**H** NMR (600 MHz, CD₃OD) δ 7.18 – 7.11 (m, 4H), 7.08 – 7.02 (m, 4H), 3.29 (m, 1H), 3.23 (t, *J* = 6.0 Hz, 4H), 2.56 (t, *J* = 6.0 Hz, 4H). ¹³C NMR (150 MHz, CD₃OD) δ 162.73, 161.10, 137.75, 137.12, 137.10, 130.86, 130.81, 129.39, 114.87, 114.73, 44.72, 27.80. **HRMS** (ESI-TOF) m/z [M+H]⁺ calculated for C₁₈H₁₈F₂N 286.1402, found 286.1404.



4-(Bis(4-fluorophenyl)methylene)-1-(but-3-yn-1-yl)piperidine (3)

The synthetic procedure was performed following previously described methods². To a solution of **2** (1.52 g, 5.3 mmol) and 3-butynyl *p*-toluenesulfonate (1.43 g, 6.36 mmol) in DMF (15 mL) was added K₂CO₃ powder (3.66 g, 26.5 mmol). The mixture was heated to 60 °C for 6 hr and then concentrated when TLC showed most of starting material being consumed. The residue was partitioned between EtOAc and water, and the aqueous layer was extracted with EtOAc. The combined organic layers were washed with sat. Na₂CO₃, dried over MgSO₄ and concentrated on rotary evaporator. The product was purified by silica gel column chromatography (0 to 5% MeOH in DCM) to yield compound **3** (1.52 g, 85%) as a yellowish oil. ¹H NMR (600 MHz, CDCl₃) δ 7.04 (dd, *J* = 8.8, 5.5 Hz, 4H), 6.95 (t, *J* = 8.8 Hz, 4H), 2.63 (dd, *J* = 8.2, 7.0 Hz, 4H), 2.53 (t, *J* = 5.6 Hz, 4H), 2.42 – 2.34 (m, 3H), 1.97 (t, *J* = 6 Hz, 1H). ¹³C NMR (150 MHz, CDCl₃) δ 162.30, 160.67, 138.07, 138.05, 135.82, 133.99, 131.28, 131.22, 115.01, 114.87, 82.70, 69.14, 56.82, 54.77, 31.47, 16.81.



4-(Bis(4-fluorophenyl)methylene)-1-(2-(1-tosyl-1H-1,2,3-triazol-4-yl)ethyl)piperidine (TH207)

The synthetic procedure was performed following previously described methods³. To a solution of **3** (0.82 g, 2.43 mmol) and TsN₃ (0.58 g, 2.92 mmol) in toluene (20 mL) was added CuTC (91 mg, 20 mol%) at room temperature and the resulting mixture was stirred for 4 hr. Upon completion monitored by TLC, the reaction mixture was concentrated and loaded for column chromatography (0 to 15% acetone in DCM) to afford compound **4** (0.69 g, 50%) as a yellowish syrup, which became solid under vacuum. ¹H NMR (600 MHz, CDCl₃) δ 8.05 – 7.87 (m, 2H), 7.36 (d, *J* = 8.3 Hz, 2H), 7.11 – 7.01 (m, 4H), 6.99 – 6.93 (m, 4H), 2.92 (t, *J* = 7.5 Hz, 2H), 2.67 (t, *J* = 7.5 Hz, 2H), 2.51 (t, *J* = 5.6 Hz, 4H), 2.43 (s, 3H), 2.36 (t, *J* = 5.6 Hz, 4H). ¹³C NMR (150 MHz, CDCl₃) δ 161.68, 160.05, 146.50, 145.55, 137.48, 135.31, 133.37, 132.62, 130.67, 130.61, 129.74, 127.95, 120.39, 114.39, 114.25, 56.23, 54.28, 30.98, 22.77, 21.17. HRMS (ESI-TOF) m/z [M+H]⁺ calculated for C₂₉H₂₉F₂N₄O₂S 535.1974, found 535.1984.



1-(2-(1H-1,2,3-Triazol-4-yl)ethyl)-4-(bis(4-fluorophenyl)methylene)piperidine (5)

To a solution of **4** (0.53 g, 1.0 mmol) in MeOH (5 mL) was added piperidine (0.43 g, 5.0 mmol) and the resulting mixture was stirred at room temperature for 0.5 hr, then concentrated and directly loaded for column chromatography (0 to 50% acetone in DCM) to afford compound **5** (0.37 g, 98%) as a yellowish syrup. ¹H NMR (600 MHz, CDCl₃) δ 7.50 (s, 1H), 7.05 (ddd, J = 8.2, 5.4, 2.2 Hz, 4H), 6.97 (td, J = 8.7, 2.3 Hz, 4H), 2.96 (td, J = 7.1, 2.2 Hz, 2H), 2.75 (td, J = 7.1, 2.1 Hz, 2H), 2.61 (td, J = 5.7, 2.2 Hz, 4H), 2.48 – 2.37 (m, 4H). ¹³C NMR (150 MHz, CDCl₃) δ 162.35, 160.71, 137.93, 137.90, 135.27, 134.41, 131.24, 131.18, 130.90, 115.06, 114.92, 109.99, 56.98, 54.83, 31.40, 21.77. HRMS (ESI-TOF) m/z [M+H]⁺ calculated for C₂₂H₂₃F₂N₄ 381.1886, found 381.1886.



4-(Prop-2-yn-1-ylcarbamoyl)benzenesulfonyl chloride (6)

Compound **6** was synthesized as reported in our previous publication⁴. ¹**H NMR** (600 MHz, CDCl₃) δ 8.13 – 7.94 (m, 4H), 7.36 (t, *J* = 5.4 Hz, 1H), 4.22 (dd, *J* = 5.4, 2.6 Hz, 2H), 2.27 (t, *J* = 2.6 Hz, 1H). ¹³**C NMR** (150 MHz, CDCl₃) δ 165.27, 146.32, 139.86, 128.66, 127.24, 78.85, 72.22, 30.02.



4-((4-(2-(4-(Bis(4-fluorophenyl)methylene)piperidin-1-yl)ethyl)-1H-1,2,3-triazol-1yl)sulfonyl)-N-(prop-2-yn-1-yl)benzamide (TH211)

To a solution of compound **5** (100 mg, 0.26 mmol) and triethylamine (TEA, 75 µL, 0.52 mmol) in anhydrous DCM (3 mL) was added a solution of compound **6** (80.2 mg, 0.31 mmol) in DCM (1 mL) at 0 °C. The resulting mixture was stirred at room temperature overnight (14 hr). The reaction mixture was concentrated and then directly loaded onto preparative TLC plate for purification (10% acetone in DCM) to yield **TH211** (55 mg, 35%, after multiple purification) as white oil, which became solid under vacuum. ¹H NMR (600 MHz, CDCl₃) δ 8.11 (d, *J* = 8.5 Hz, 2H), 8.01 (s, 1H), 7.98 (d, *J* = 8.5 Hz, 2H), 7.03 (dd, *J* = 8.5, 5.6 Hz, 4H), 6.95 (dd, *J* = 9.9, 7.5 Hz, 5H), 4.21 (dd, *J* = 5.3, 2.6 Hz, 2H), 2.93 (d, *J* = 7.4 Hz, 2H), 2.69 (t, *J* = 7.4 Hz, 2H), 2.53 (s, 4H), 2.36 (t, *J* = 5.6 Hz, 4H), 2.24 (t, *J* = 2.6 Hz, 1H). ¹³C NMR (150 MHz, CDCl₃) δ 187.96, 164.87, 162.32, 160.69, 140.22, 138.72, 137.92, 131.24, 131.18, 128.79, 128.59, 121.36, 115.05, 114.90, 78.79, 72.20, 56.60, 54.82, 31.38, 29.97, 23.17. HRMS (ESI-TOF) m/z [M+H]⁺ calculated for C₃₂H₃₀F₂N₅O₃S 602.2032, found 602.2032.



4-(Prop-2-yn-1-yloxy)benzenesulfonyl chloride (7)

The synthesis of compound 7 was adapted from a previously reported method⁵. Briefly, to a solution of phenyl propargyl ether (1.01g, 7.5 mmol) in DCM (15 mL) was added chlorosulfonic acid (3.46 g, 30 mmol) dropwise using glass syringe at 0 °C ice bath. The black mixture was stirred for 15 min, then poured into ice water to quench the reaction. The aqueous layer was extracted twice with DCM. The combined organic layers were washed with brine, dried over MgSO₄, and concentrated to get a greenish oil, which was purified by column chromatography (20% EtOAc in hexane) to afford compound 7 (1.08 g, 62%) as a yellowish oil. ¹H NMR (600 MHz, CDCl₃) δ 7.98 (d, *J* = 9.1 Hz, 2H), 7.13 (d, *J* = 9.1 Hz, 2H), 4.80 (d, *J* = 2.4 Hz, 2H), 2.60 (t, *J* = 2.4 Hz 1H). ¹³C NMR (150 MHz, CDCl₃) δ 162.62, 136.86, 129.46, 115.62, 77.04, 76.86, 56.30.



4-(Bis(4-fluorophenyl)methylene)-1-(2-(1-((4-(prop-2-yn-1-yloxy)phenyl)sulfonyl)-1H-1,2,3triazol-4-yl)ethyl)piperidine (TH214)

TH214 was synthesized from compound **5** and **7** using the same method as **TH211**. ¹**H NMR** (600 MHz, CDCl₃) δ 8.07 – 8.00 (m, 2H), 7.97 (s, 1H), 7.12 – 7.07 (m, 2H), 7.06 – 7.00 (m, 4H), 7.00 – 6.91 (m, 4H), 4.75 (d, *J* = 2.4 Hz, 2H), 2.98 (d, *J* = 7.5 Hz, 2H), 2.76 (s, 2H), 2.62 – 2.57 (m, 4H), 2.56 (m,1H), 2.42 (t, *J* = 5.5 Hz, 4H). ¹³**C NMR** (150 MHz, CDCl₃) δ 162.96, 162.29, 160.66, 146.09, 138.08, 138.06, 135.93, 133.99, 131.27, 131.22, 131.02, 128.16, 120.88, 115.83, 115.01, 114.87, 77.03, 76.77, 56.86, 56.21, 54.90, 31.61, 23.39. **HRMS** (ESI-TOF) m/z [M+H]⁺ calculated for C₃₁H₂₉F₂N₄O₃S 545.1818, found 545.1812.



4-Iodobenzensulfonic Acid (9)

The synthetic procedure was performed following previously described methods⁶. To iodobenzene (2 mL, 3.64 g, 17.8 mmol) was added concentrated sulfuric acid (5 mL, 8.0 g, 82 mmol) and the reaction mixture was heated to 50 °C for 30 hr. Then the reaction mixture was stirred with hexane (5 mL) for 5 min in order to remove unreacted iodobenzene in the hexane layer. The sulfuric acid layer was extracted with small portions of boiling chloroform (total 20 mL) by removing the upper layer (solution of product in CHCl₃) with a pipette. The combined chloroform solution was concentrated to a small volume, and precipitates of the product were collected, washed with hexane, and dried to afford 4-iodobenzensulfonic acid (4.0 g, 80%), which was used for the next step without further purification.

4-((Trimethylsilyl)ethynyl)benzenesulfonic acid (10)

The synthetic procedure was performed following previously described methods⁷. To a solution of 4-iodobenzensulfonic acid (2.22 g, 7.8 mmol) in anhydrous DMF (20 mL) was added trimethylsilylacetylene (6.6 mL, 47 mmol), triethylamine (10 mL, 78 mmol), PdCl₂(PPh₃)₂ (550 mg, 0.78 mmol) and CuI (75 mg, 0.78 mmol), and the mixture was stirred at room temperature overnight (14 hr). After being concentrated under rotary evaporator, the residue was purified on flash silica gel column chromatography (10% MeOH in DCM) to afford the compound **10** (as form of triethylammonium salt, 2.07 g, 74%). ¹**H NMR** (600 MHz, CDCl₃) δ 7.79 – 7.63 (m, 2H), 7.38 (d, *J* = 8.2 Hz, 2H), 3.01 (ddd, *J* = 7.4, 4.8, 1.0 Hz, 6H), 1.35 – 1.04 (m, 9H), 0.16 (d, *J* = 0.8 Hz, 9H). ¹³**C NMR** (150 MHz, CDCl₃) δ 145.21, 131.66, 125.74, 124.57, 104.21, 95.66, 46.35, 8.65, -0.17.

4-Ethynylbenzenesulfonic acid (11)

To a solution of compound **10** (568 mg, 1.60 mmol) in THF was added tetrabutylammonium fluoride (1.92 mL, 1 M solution in THF), and the mixture was stirred at room temperature for 2 hr. After being concentrated under rotary evaporator, the crude product was used for the next step without further purification.

4-Ethynylbenzenesulfonyl chloride (8)

To a solution of crude **11** (1.6 mmol) in DMF (3 mL) was added thionyl chloride (37 μ L, 8 mmol) at 0 °C. The mixture was stirred at room temperature for 3 hr and then poured into ice water to quench unreacted thionyl chloride. The mixture was extracted with EtOAc for several times and the combined organic layers were washed with brine, dried over MgSO₄ and concentrated under rotary evaporator. The residue was purified on flash silica gel column chromatography to afford compound **8** (96 mg, 30% over two steps) as yellowish solid, which was immediately frozen to suppress the hydrolysis of sulfonyl chloride. ¹H NMR (600 MHz, CDCl₃) δ 7.98 (dd, *J* = 8.6, 1.7 Hz, 2H), 7.68 (dd, *J* = 8.6, 1.7 Hz, 2H), 3.37 (s, 1H). ¹³C NMR (150 MHz, CDCl₃) δ 143.71, 133.13, 129.58, 126.95, 82.81, 81.31.



4-(Bis(4-fluorophenyl)methylene)-1-(2-(1-((4-ethynylphenyl)sulfonyl)-1H-1,2,3-triazol-4yl)ethyl)piperidine (TH216)

TH216 was synthesized from compound **5** and **8** using the same method as **TH211**. ¹**H NMR** (600 MHz, CDCl₃) δ 8.04 (d, *J* = 8.6 Hz, 2H), 7.98 (s, 1H), 7.64 (d, *J* = 8.5 Hz, 2H), 7.04 (dd, *J* = 8.6, 5.6 Hz, 4H), 6.96 (t, *J* = 8.7 Hz, 4H), 3.34 (s, 1H), 3.00 (s, 2H), 2.76 (s, 2H), 2.60 (s, 4H), 2.42 (s, 4H). ¹³**C NMR** (150 MHz, CDCl₃) δ 162.35, 160.72, 137.89, 137.87, 135.83, 133.16, 131.22, 131.17, 129.88, 128.46, 121.20, 115.07, 114.93, 82.79, 81.25, 56.64, 54.81, 31.24, 23.10. **HRMS** (ESI-TOF) m/z [M+H]⁺ calculated for C₃₀H₂₇F₂N₄O₂S 575.1923, found 575.1924.

3. BIOLOGICAL METHODS

Reagents

Ritanserin (purity >95%), Staurosporine (purity >98%) and ATP (disodium salt hydrate, purity >98%) used in competition studies were purchased from Cayman Chemical Company, Fisher Scientific and Acros, respectively.

Cell culture

Jurkat cells were cultured in RPMI media 1640 (Gibco) and HEK293T cells were in DMEM, both supplemented with 10% fetal bovine serum (FBS, U.S. Source, Omega Scientific) and 1% L-glutamine (Thermo Fisher Scientific). SILAC cells were cultured with corresponding SILAC media supplemented with 10% dialyzed FBS and either ¹²C, ¹⁴N-lysine and arginine (100 μ g/mL) for "light" or ¹³C, ¹⁵N-lysine and arginine (100 μ g/mL) for "heavy". All cells were maintained in a humidified incubator at 37 °C with 5% CO₂, and used for experiments around 90% confluency (HEK293T) or 1×10⁶ cells/mL (Jurkat).

Transient transfection

HEK293T cells for overexpression studies were cultured at 37 °C with 5% CO2 in DMEM media (HyClone) with 10% fetal bovine serum (Omega Scientific), 1% L-glutamine (Fisher Scientific). The recombinant proteins were generated as previously described via transient transfection⁸. The wild-type human PKC-α plasmid was purchased from GenScript: pcDNA3.1-PRKCA-FLAG

(NM_002737.2). The wild-type human DGK α plasmid was generated in house: pGC-FLAG-DGKA.

Phorbol-12-myristate-13-acetate (PMA) activation of cells

PMA (EMD Millipore) was dissolved in DMSO to form a 500 μM solution, which was then diluted in serum free DMEM media (1:1,000, 500 nM final).48 hours after transfection, the cell culture media was removed. The cells were starved for 30 min at 37 °C with 5% CO₂ in serum free medium. The PMA was added to the final concentration of 500nm and cells were treated with PMA for 6 hours. After the PMA treatment, cells were washed and lysed in NP40 lysis buffer (50 mM Tris-HCl pH: 7.5, 1% NP40, 150 mM NaCl plus protease and phosphatase inhibitor). The cell lysates were sonicated and centrifuged for 20 minutes at 13000 rpm. Protein concentrations were determined using Bio-Rad DC kit. The degradation rate of both wild type and mutant PRKCA proteins were analyzed using western blot⁴.

Western blot analysis

The supernatant after centrifugation was collected as the soluble fraction. The pelleted residue was resuspended in lysis buffer supplemented with 1% Triton X-100 followed by centrifugation at $17,000 \times g$ for 10 minutes. The supernatant was collected as the membrane fraction. Protein concentrations were determined using Bio-Rad DC protein assay and adjusted to 1 mg/mL in PBS followed by Western blot analysis as previously described⁴.

Site-directed mutagenesis

Mutated site	Primers	Sequence
h <i>DGK</i> p.Y240F	hDGKAp.Y240F Fr	actggtcgtgaacagtgaacttacagaggttacag
	hDGKAp.Y240F Re	ctgtaacctctgtaagttcactgttcacgaccagt
hPRKCAp.K209A	hPRKCAp.K209A Fr	cggatggttttggttttttgcgcgctttcattcttgggatcagg
	hPRKCAp.K209A Re	cctgatcccaagaatgaaagcgcgcaaaaaaaccaaaaccatccg
hPRKCApY195F	h <i>PRKCA</i> pY195F-Fr	caaacgggctttcagatccttttgtgaagctgaaacttat
	hPRKCApY195F-Re	ataagtttcagcttcacaaaaggatctgaaagcccgtttg
hPRKCApK232A	hPRKCApK232A-Fr	gtcggtctttgtctgaaggtgccaatttgaatgtaaaggactcattcc
	hPRKCApK232A-Re	ggaatgagtcetttacattcaaattggcacettcagacaaagacegac

To introduce the mutations in *DGKA* and *PRKCA*, pcDNA3.1 vectors containing the cDNA of human *DGKA* or human *PRKCA* with a C-terminal flag signal were purchased from GenScript (<u>www.genscript.com</u>). To introduce three mutations in *PRKCA* and one mutation in *DGKA*, several primers were designed (see table above). Different mutations were introduced using site directed mutagenesis method (Quickexchange kit, Agilent Technologies). The sequence of each clone was confirmed using sanger sequencing.

Live cell treatment with probes and cell lysate preparation

Cells were treated with DMSO or probes (2.5 mM in DMSO, 50X, final concentration at 50 μ M) in serum-free media for 2 hr at 37 °C with 5% CO₂, and then collected, washed with cold PBS for three times. For competition studies, cells were pretreated with vehicle (DMSO) or compound (50X stock in DMSO) at the same condition for 1 hr prior to probe treatment. The cell pellets were lysed by sonication (1 sec pulse, 20% amplitude, 3 times) in PBS in the presence of EDTA-free protease inhibitor cocktail tablet (Pierce). The cell lysates were subject to ultracentrifugation (100,000 x g, 45 min at 4 °C) to separate the cytosolic fraction in the supernatant and the membrane fraction as a pellet. The membrane pellet was re-suspended in the protease inhibitor-containing PBS by sonication. Protein concentrations in both fractions were measured by the Bio-Rad DC protein assay and adjusted to levels as needed.

Gel-based chemical proteomic assay

Proteome aliquots (2 mg/mL, 49 μ L) were treated with probes at indicated concentration (1 μ L, 50X stock in DMSO) at 37 °C for 1 hr. The subsequent conjugation with fluorophore was accomplished by copper-catalyzed azide-alkyne cycloaddition (CuAAC) with rhodamine-azide (TAMRA-azide, 1.25 mM, 1 μ L, final concentration of 25 μ M) in the presence of tris(2-carboxyethyl)phosphine (TCEP, 50 mM fresh in water, 1 μ L, final concentration of 1 mM), tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA, 1.7 mM in 4:1 *t*-butanol/DMSO, 3 μ L, final concentration of 100 μ M) and CuSO₄ (50 mM, 1 μ L, final concentration of 1 mM). For samples of live cell treatment, it started with click reaction without the step of the probe treatment *in vitro*. After 1 hr incubation at room temperature, reactions were quenched by adding 4X SDS-PAGE loading buffer and beta-mercaptoethanol (17 μ L) and samples were resolved by SDS-PAGE followed by in-gel fluorescence scanning.

SILAC sample preparation for MS-based chemical proteomic assay

The light proteome aliquots (2.3 mg/mL, 432 μ L) were treated with the probe (5 μ L, 100X fresh stock in DMSO) and the heavy proteome aliquots were treated with either the same probe or DMSO (no probe) at 37 °C for 1 hr. Samples were subjected to click reaction with the desthiobiotin-PEG3-azide (10 mM in DMSO, 10 μ L, final concentration 200 μ M) in the presence of TCEP (50 mM, 10 μ L), TBTA (1.7 mM, 33 μ L) and CuSO₄ (50 mM, 10 μ L) at room temperature for 1 hr. The light and the heavy samples were mixed in the chloroform-methanol extraction step. The subsequent steps including reduction with dithiothreitol, alkylation with iodoacetamide, digestion with Trypsin/Lys-C, enrichment with avidin beads were conducted as previously described^{4, 9}.

Competitive chemical proteomics

For *in vitro* studies, the light and heavy proteomes were pretreated with vehicle (DMSO) or the inhibitor/ligand, respectively, at 37 °C for 1 hr prior to probe treatment for another 1 hr. For *in situ* studies, the light and heavy proteomes prepared from cells that were pretreated *in situ* with vehicle (DMSO) or the inhibitor, respectively, at 37 °C for 1 hr followed by probe treatment for 2 hr, were directly subjected to click reaction following the steps described above.

LC-MS/MS analysis

Data acquisition: The enriched peptide samples were analyzed by LC-MS/MS using an EasynLC 1200 (Thermo Scientific) coupled with an Orbitrap Q Exactive Plus mass spectrometer (Thermo Scientific). A trap column (Acclaim PepMapTM, Thermo Scientific, 75 μ m × 2 cm, 3 μ m C18) and a homemade nanocapillary analytical column (20 cm, 5 μ m C18 packed in 360 μ m O.D. × 75 μ m I.D. fused silica) with an integrated electrospray tip were employed with the following LC gradient (mobile phase A: 0.1% formic acid in H₂O; mobile phase B: 80% ACN, 0.1% formic acid in H₂O): 0 – 1.48 min 1% B, 400 nL/min; 1.48 – 2 min 1% B, to 300 nL/min; 2 – 10 min to 13% B, 300 nL/min; 10 – 110 min to 32% B, 300 nL/min; 110 – 151 min to 60% B, 300 nL/min; 151 – 152 min to 95% B, 300 nL/min; 152 – 160 min, 95% B, 300 nL/min; 160 – 161 min to 1% B, 300 nL/min; 161 – 161.1 min 1% B, to 400 nL/min; 161.1 – 180 min 1% B, 400 nL/min. A top 10 data-dependent acquisition method was used, which consisted of one full scan MS1 scan (m/z 375 – 1,500) followed by ten MS2 scans of the most abundant ions recorded in the MS1 scan.

Data analysis: Identification of peptides and target proteins from the LC-MS/MS raw data was achieved using the Byonic software package (Protein Metrics Inc.) to search against a modified human protein database (UniProt human protein database, angiotensin I and vasoactive intestinal peptide standards; 40660 proteins) with parameters previously described.⁴ Identification of probe-modified peptides was accomplished with variable (common) modification of +635.27374 Da for TH211, +608.26284 for TH214, +578.25228 for TH216 on tyrosine and lysine residues. Fixed cysteine carbamidomethylation (+57.02146 Da) and variable modifications of methionine oxidation (+15.9949 Da), and the SILAC heavy amino acids (+10.0083 Da for R, +8.0142 Da for K) were included in search. The results from Byonic searches were imported into Skyline-daily to determine SILAC ratio (SR) of light/heavy peptide abundances⁹. To account for variations in

mixing and sample preparation, SRs of peptides from probe/no-probe (the light was treated with the probe and the heavy was treated with DMSO) were normalized to those from probe/probe (both the light and the heavy were treated with the probe). Unless otherwise stated, the results from Skyline-daily output were filtered in R to keep peptides of high quality as determined by the following criteria: Byonic score \geq 300; a precursor mass error within 5 ppm; normalized SR \geq 5, with both isotope dot-product (iDOTP) and ratio dot-product (rDOTP) \geq 0.8. Comparison of probe-modified sites across all probes and cells was performed using the R package ggplot2 and Venn diagrams were generated with the VennDiagram R package⁴. Other bioinformatics analysis such as the domain enrichment analysis were performed as previously described⁴. For analysis of kinases, we also manually verified the identity of the following probe-modified peptides that did not meet our quality control criteria described above: Q53H12 (AGK, Y224), Q8TBX8 (PI42C, K384), Q9HA64 (KT3K, K45), Q96S44 (PRPK, Y67), and P27361 (MK03, Y280). Annotated MS/MS spectra that support identification of probe-modified peptides are included in the Appendix.

The analysis of TH211-modified peptides from human DGK α revealed that Y240 and Y623 appeared to be enriched for detection in membrane compared with soluble fractions. Detection of probe modification of the Y22 site of the RVH domain was enriched in soluble fraction analyses. Probe modification of Y544 appeared to be detected to an equivalent degree in both fractions.

Biochemical substrate assay of DGKa

The liposomal method for measuring purified DAG kinase activity was similar to previously described methods^{8, 10}. Transient transfections with human DGK α wild-type or Y240F mutant were performed using PEI (Polysciences, Warrington, PA). Cells were fed with fresh media 24 hrs after transfection, and 48 hr following the transfection, the cells were harvested and homogenized with a 22-G needle using 500 µL/plate of lysis buffer (20 mM HEPES, pH 7.2, 150 mM NaCl, 0.5 mM DTT, 0.1% Brij-35, and the protease inhibitors phenylmethylsulfonyl fluoride (PMSF), leupeptin, and pepstatin). The cell homogenates were cleared by centrifugation at 16,000 x *g* for 10 min at 4°C. The supernatant was collected and incubated with 15 µL/plate of FLAG (M2) beads for 2 hr at 4 °C. The beads were then loaded on an affinity screening column (Fisher Scientific, Waltham, MA) and washed 3 times with lysis buffer, followed by 3 washes with wash buffer (20 mM HEPES, 150 mM NaCl, pH 7.2). The FLAG-DGK α was eluted with 5 successive additions of equal volume of 0.5 mg/mL of FLAG peptide. The fractions were collected and pooled, and purified enzyme was visualized on an SDS-PAGE gel stained with Coomassie-blue dye. The protein yield was quantified by comparison to bovine serum albumin (BSA) standards.

Lipids were prepared for liposome formation by dissolving dioleoyl phosphatidylcholine (DOPC), dioleoyl phosphatidylserine (DOPS), and dioleoyl DAG (DODG) in chloroform, combining, and drying under nitrogen gas to remove all solvent. The surface concentration of lipids within the liposomes was 10 mol% DAG, 20 mol% DOPS, and 70 mol% DOPC. The lipids were then hydrated to 10 mM in liposome buffer (50 mM (3-(*N*-morpholino))propanesulfonic acid) (MOPS), pH 7.5, 100 mM NaCl and 5 mM MgCl₂). The hydrated lipids were subjected to five freeze-thaw cycles in liquid nitrogen, followed by extrusion through a 100 nm polycarbonate filter 11 times. The measurement of DGK activity was determined by following the incorporation of the γ -Phosphate from [γ -³²P]ATP into DAG to form a radiolabeled PA product. Immediately prior to

running assays, $[\gamma^{-32}P]ATP$ was prepared by adding 0.5 – 2 µL of $[\gamma^{-32}P]ATP$ (6000 Ci/mmol) to 500 µL of 10 mM ATP. The assays were run as 100 µL reactions and contained liposome buffer, 0.1 mM CaCl₂, 1 mM dithiothreitol (DTT), appropriate purified enzyme as indicated in figure legends, 20 µL liposomes (2 mM lipids), and were initiated with the addition of 10 µL of the prepared 10 mM $[\gamma^{-32}P]$ ATP. The reactions were allowed to proceed for 20 min at 30 °C before being terminated with the addition of 0.5 mL methanol with 0.1 N HCl, followed by 1 mL of ethyl acetate. Phase separation was achieved with the addition of 1 mL 1 M MgCl₂, and the organic phase was washed by thoroughly vortexing to remove soluble $[\gamma^{-32}P]$ ATP. To measure the incorporation of $[^{32}P]$ into DAG, the extract was centrifuged at 200 x G for 1 min, and 0.5 mL of the organic phase was removed and the radioactivity was measured using a scintillation counter. The activity of each purified DGK was normalized to background extracts without enzyme added. Activity was calculated as nmol of PA produced per min per µg of purified enzyme present in the assay.

4. REFERENCES

- 1. J. Lee, S. U. Kang, J. O. Lim, H. K. Choi, M. K. Jin, A. Toth, L. V. Pearce, R. Tran, Y. Wang, T. Szabo and P. M. Blumberg, *Bioorg Med Chem*, 2004, **12**, 371-385.
- 2. S. C. Turner, T. A. Esbenshade, Y. L. Bennani and A. A. Hancock, *Bioorg Med Chem Lett*, 2003, **13**, 2131-2135.
- 3. J. Raushel and V. V. Fokin, *Org Lett*, 2010, **12**, 4952-4955.
- 4. H. S. Hahm, E. K. Toroitich, A. L. Borne, J. W. Brulet, A. H. Libby, K. Yuan, T. B. Ware, R. L. McCloud, A. M. Ciancone and K. L. Hsu, *Nat Chem Biol*, 2020, **16**, 150-159.
- 5. M. Kheirabadi, G. S. Creech, J. X. Qiao, D. S. Nirschl, D. K. Leahy, K. M. Boy, P. H. Carter and M. D. Eastgate, *J Org Chem*, 2018, **83**, 4323-4335.
- 6. M. S. Yusubov, R. Y. Yusubova, V. N. Nemykin, A. V. Maskaev, M. R. Geraskina, A. Kirschning and V. V. Zhdankin, *European Journal of Organic Chemistry*, 2012, 2012, 5935-5942.
- 7. S. Paoletta, D. K. Tosh, A. Finley, E. T. Gizewski, S. M. Moss, Z. G. Gao, J. A. Auchampach, D. Salvemini and K. A. Jacobson, *J Med Chem*, 2013, **56**, 5949-5963.
- 8. T. B. Ware, C. E. Franks, M. E. Granade, M. Zhang, K. B. Kim, K. S. Park, A. Gahlmann, T. E. Harris and K. L. Hsu, *Nat Chem Biol*, 2020, **16**, 170-178.
- 9. C. E. Franks, S. T. Campbell, B. W. Purow, T. E. Harris and K. L. Hsu, *Cell Chem Biol*, 2017, **24**, 870-880 e875.
- 10. S. Boroda, M. Niccum, V. Raje, B. W. Purow and T. E. Harris, *Biochem Pharmacol*, 2017, **123**, 29-39.

5. APPENDIX

5.1 NMR Spectra























































5.2 MS/MS Spectra of probe-modified peptides















