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Supplementary Figures

Supplementary Figure 1. Gel-based ABPP of the membrane (mem), soluble (sol), and secreted (CM) fractions of NOTUM- or METAP2 (mock)-transfected HEK293T cells or SW620 cells. Gel-based ABPP was performed by incubating samples with FP-Rh (1 µM, 30 min, room temperature). Competitive ABPP shown in the rightmost gel lane for ABC28 was performed by pretreating the SW620 CM with ABC28 (10 µM, 37 °C, 30 min) prior to FP-Rh treatment.
Supplementary Figure 2. Structure-activity relationship (SAR) and selectivity of NHH carbamate inhibitors of NOTUM. (A, B) Structures (A) and inhibitory activity (B) of chlorine positional scanning analogues of ABC28 – ABC90, ABC91, and ABC92. The inhibitory
profiles were measured using the CM of SW620 cells as a source of endogenous NOTUM and the indicated concentrations of inhibitors (30 min pre-incubation, 37 °C) followed by treatment with FP-Rh (1 µM, 30 min, room temperature), prior to SDS-PAGE analysis and in-gel fluorescence scanning. (C) Competitive ABPP of SW620 cells treated in situ with ABC90 (indicated concentrations, 2 h, 37 °C), followed by harvesting, lysis, subcellular fractionation, treatment with FP-Rh (1 µM, 30 min, room temperature) and SDS-PAGE analysis. (D) ABPP analysis of the whole cell lysates from ABC90- or ABC63-treated cells, using the PPT1-directed click probe ABC45. Cell lysates were treated with ABC45 (1 µM, 30 min, RT), followed by PNGaseF to deglycosylate PPT1 (30 min, 37 °C), CuAAC conjugation with a rhodamine-azide (Rh-N3) tag, and SDS-PAGE analysis.
Supplementary Figure 3. Quantitative MS-based ABPP of SW620 cells treated in situ (2 h, 37 °C) with ABC99 (0.5 or 10 µM) or ABC101 (10 µM). After compound treatment, cells were harvested, lysed, and serine hydrolases enriched with FP-biotin (4 µM, 1 h, room temperature) and streptavidin chromatography. After on-bead tryptic digestion, peptides were isotopically labeled with heavy (DMSO) or light (compound treated) formaldehyde, then combined and processed for MS-based analysis. Ratios are displayed as light/heavy; therefore, low values indicate inhibition. Data for each experimental group represent median aggregate peptide ratios across two independent experiments ± S.E.M.
**Supplementary Figure 4.** ABPP gel showing competitive blockade of FP-Rh labeling (left lanes) and direct protein labeling (right lanes) by ABC99yne in SW620 cell lysates. Samples were pretreated with ABC99yne at the indicated concentrations (30 min, 37 °C) and then treated with FP-Rh (1 µM, 30 min, room temperature) or subjected to CuAAC conditions with an Rh-N₃ tag prior to SDS-PAGE analysis.
**Biological Methods**

The following cell lines were obtained from ATCC: HEK293T (CRL3216), HEK293-STF (CRL-3249), SW620 (CRL-227), L (CRL-2648), L-Wnt3A (CRL-2647) and grown according to instructions from ATCC.

NOTUM cDNA was obtained from GE Dharmacon (Catalog #MHS6278-202806033) and subcloned into the pRK5 vector bearing a CMV promoter and C-terminus FLAG tag. The NOTUM S232A mutation was generated using the QuikChange II Site Directed Mutagenesis Kit (Agilent).

Compounds used in initial screening (Figure 1C): AA26-7, AA26-8, AA26-9, AA39-2, AA39-3, AA44-2, AA74-1, KT109, KT116, KT117, KT185, KT195, KT205, KLH45, ABC5, ABC23, ABC34, ABC44, ABC45, and ABC47 were synthesized as previously described (AA compounds², KT compounds³, KT185⁴, KLH45⁵, ABC compounds¹).

**Transient Overexpression in HEK293T cells**

HEK293T cells were seeded at $6 \times 10^6$ cells per 15 cm tissue culture plate and grown for 48 hours. Cells were then transfected with NOTUM, NOTUM S232A, or the control protein METAP2. For each transfection, 10 µg of each construct was incubated with 30 µL PEI MAX (1 mg/mL) in 1 mL of serum-free DMEM for 30 minutes, before being added to cells. Conditioned media was then collected as described below.
Preparation of Conditioned Media

Conditioned media was collected from HEK293T cells transiently overexpressing NOTUM, NOTUM S232A or the control protein METAP2. Media was removed 24 hours following transfection and cells were washed two times with warm PBS, and serum-free DMEM supplemented with 1 x PenStrep Glutamine was added. Media was collected on ice 48 hours later, cellular debris was pelleted (2,800g, 5 minutes) and the supernatant was applied to a 10,000 MWCO filter (EMD Millipore UFC701008) and concentrated at 4 °C (3,500g, ~30 minutes). One volume of cold PBS was then added to the concentrated media and the centrifugation step was repeated. Conditioned media was aliquoted, snap frozen, and stored at -80 °C for later use.

Conditioned media from SW620 cells was collected as above with the following modifications. SW620 cells were seeded at 5 × 10^6 per 15 cm plate, and allowed to grow to 60-80% confluency. Cells were then washed twice with warm PBS, and serum-free RPMI supplemented with 1 x PenStrep Glutamine was added. Media was collected 48 hours later and processed as described above.

Gel-based ABPP

In vitro and in situ competitive gel-based ABPP was performed as described previously.\(^3\,6\)

Briefly, after in vitro (30 min, 37 °C) or in situ (2 hours, 37 °C) inhibitor or DMSO treatment, cell lysates were treated with 1 µM FP-Rhodamine (FP-Rh) probe for 30 minutes at room temperature. Samples were then quenched with 4X SDS loading buffer, separated by SDS-PAGE on a 10% acrylamide gel and in-gel fluorescence was imaged using a ChemiDoc MP system.
Western Blotting

Following SDS-PAGE, gel samples were transferred onto nitrocellulose (50V, 2 hours), and blots were blocked with 5% milk TBS-T (30 min, RT), then incubated with anti-NOTUM antibody (Sigma-Aldrich HPA023041, 1:1000) overnight at 4°C. Blots were then washed with TBS-T (3x) and incubated with a secondary antibody (Li-cor IRDye 800CW Donkey anti-rabbit, 1:5000 in TBS-T with 5% milk) for 2 hours. Transfers were then washed again (3x, TBS-T) and imaged on a Li-cor Odyssey (Model 9120).

PPT1 Activity Profiling

Gel-based determination of PPT1 inhibition was carried out as described previously.\textsuperscript{1} Briefly, SW620 cells were grown in 6 cm plates to ~80% confluency, and then treated with inhibitors or DMSO \textit{in situ} for 2 hours. Cells were then scraped on ice, washed once with cold PBS, snap frozen and stored at -80 °C. Cell pellets were then thawed on ice, re-suspended in cold PBS and lysed with a probe sonicator. The lysates were pre-cleared (1,400g, 1 min) and the supernatant was fractionated by ultracentrifugation (100,000g, 45 min, 4 °C). The soluble fraction was collected, and sample concentrations were adjusted to 2 mg/mL. Samples were then incubated with 1 µM of the PPT1 probe \textbf{ABC45} for 30 minutes at room temperature, and for an additional 30 minutes at 37 °C following addition of the de-glycosylating enzyme PNGaseF. A rhodamine-azide (Rh-N\textsubscript{3}) fluorophore reporter was conjugated onto the alkyne probe using CuAAC conditions as reported previously.\textsuperscript{7}
**ABC99yne Labeling**

Proteome derived from either SW620 whole cell lysates (1 mg/mL), or from SW620 conditioned media (0.1 mg/mL) was treated with varying concentrations of ABC99yne for 30 minutes at room temperature, followed by clicking the fluorophore reporter rhodamine azide (Rh-N₃) with conditions as described above for PPT1 Activity Profiling.

**Mass-spectrometry ABPP Sample Preparation and Data Analysis**

Samples for quantitative mass spectrometric analysis were prepared and analyzed as previously reported with minor modifications specified below. Inhibitor and DMSO treated proteomes from SW620 whole cell lysates (1 mg per condition), or SW620 conditioned media (0.5-0.75 mg per condition) were treated with 4 µM FP-biotin for 1 hour at room temperature. Samples were then precipitated using chloroform/methanol, reduced with 10 mM neutral TCEP (30 minutes, 37 °C), alkylated with 40 mM iodoacetamide (30 minutes, room temperature), before being enriched with PBS-washed streptavidin-agarose beads (100 µL slurry, Thermo) for 1.5 hours at room temperature with end-over-end rotation. Samples were then washed with 0.2% SDS (2 x 10 mL), transferred to Low-bind eppendorf tubes, washed with 3 x 1 mL PBS, and 3 x 1 mL DI H₂O, and then digested with sequence-grade trypsin (2 µg, Promega) in 2M urea overnight. Samples were then labeled using reductive dimethylation (ReDiMe) as previously described.

Samples as prepared above were analyzed by LC/MS on a Thermo Finnigan instrument using a five-step multidimensional LC/MC MudPIT protocol, with conditions as previously described.
Spectrum raw files were extracted into MS2 files using RawConverter (http://fields.scripps.edu/rawconv/) and searched using the ProLuCID algorithm against a human reverse concatenated nonredundant Uniprot database, with static modifications for cysteine residues to account for alkylation by iodoacetamide (+57.0215 m/z), and standard static modifications for ReDiMe: lysine and N-terminus (+28.0313 m/z for light, +34.06312 m/z for heavy). Data was assembled using DTASelect version 2.0, and ratio quantification was performed using in-house CIMAGE software. Peptides were required to have an envelope correlation score of $R^2 \geq 0.8$. Half tryptic peptides were discarded and ratios were capped to a maximum value of 20. Replicate data was combined with the requirement that proteins are quantified in at least two replicates. In cases where proteins had exactly one peptide with a calculated ratio of 20, and at least one other peptide with a ratio below 4, the 20 value was discarded.

**Wnt Activity Assay**

Wnt activity was assayed using the HEK293-STF cell line as described previously, with some modifications. SW620 cells were seeded at a concentration of $5 \times 10^6$ in 10 cm plates. Two days later, media was collected, sterile filtered through a 22 µm syringe filter, and immediately incubated with inhibitors or DMSO for 1 hour at 37 °C, prior to being mixed 1:1 with freshly collected media from L, or L-Wnt3A cells (seeded at $2 \times 10^6$ per 10 cm plate, and processed the same way as media from SW620 cells). The mixture was then incubated at 37 °C for two hours, before being added to HEK293-STF (seeded at $3 \times 10^4$ per well of a 96-well plate 24 hours earlier).
Luminescence was then measured 24 hours later following replacement of media with 80 µL of Bright-Glo reagent (BioRad). Luminescence measurements for each condition were performed in quadruplicate.

**Chemical Methods**

Chemicals and solvents were purchased from reputable vendors and used without further purification. $^1$H NMR spectra were obtained on a Bruker 500 mHz. Coupling constants (J) are reported in hertz. HRMS service was performed by the TSRI Center for Mass Spectrometry using an Agilent ESI-TOF instrument. All compounds were prepared as racemates.

1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-(4-methoxyphenyl)piperazine1-carboxylate and tert-butyl 2-hydroxy-1,3-dioxohexahydroimidazo[1,5-a]pyrazine-7(1H)-carboxylate were prepared as previously described.$^1$

**General Procedures**

**General Procedure A. Reductive Amination.**

$$
\text{X=N-O-C=O} + \text{O-C=O} \rightarrow \text{X-N-C=O-O-C=O}
$$

To a stirring solution of the amine containing compound in DCM (0.1 M, 1 eq), the aldehyde was added (4 eq), followed by sodium triacetoxyborohydride (2 eq), and finally acetic acid (catalytic, ~0.01 eq). The resulting solution was stirred overnight at room temperature, concentrated to a residue, and separated by prep-TLC.

**General Procedure B. Boc Deprotection.**
A 2 M solution of methanolic HCl was prepared by adding acetyl chloride to cold methanol, dropwise. After warming to room temperature, the Boc-protected amine was dissolved in this solution at a concentration of ~0.1 M. The resulting solution was stirred for 30 min at room temperature, then neutralized with sodium bicarbonate, and extracted with ether or DCM. The solvent was evaporated under nitrogen, and the resulting amine was dried on the high-vac and used without further purification.

Synthesis of NOTUM Inhibitors

\((2,3\text{-dihydro-4H-benzo}[b][1,4]\text{oxazin-4-yl})(1\text{H-imidazol-1-yl})\text{methanone (ABC99}\_\text{IU})\)

To a stirring solution of carbonyldiimidazole (Combi-Blocks) (1080 mg, 6.66 mmol) of DCM (4.5 mL) on ice 3,4-Dihydro-2H-1,4-benozxazine (Combi-Blocks) (600 mg, 4.44 mmol) was slowly added, dropwise. The solution was warmed to room temperature and stirred for 6 hours, then washed 4x with H\(_2\)O, filtered through sodium sulfate, concentrated to a residue, and purified by flash chromatography on SiO\(_2\) (EtOAc) to yield the imidazole urea \textbf{ABC99}\_\text{IU} as a white solid (873 mg, 86%). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 7.90 (m, 1H), 7.18 (t, \(J = 1.4\) Hz, 1H), 7.06 (m, 2H), 6.96 (m, 1H), 6.78 (m, 2H), 4.43 (m, 2H), 4.00 (m, 2H). HRMS calculated for C\(_{12}\)H\(_{11}\)N\(_3\)O\(_2\) [M+H]\(^+\) 230.0924, found 230.0928.

\(7\text{-}(\text{tert-butoxycarbonyl})\cdot1,3\text{-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 2,3-dihydro-4H-benzo[b][1,4]oxazine-4-carboxylate (preABC99}\_\text{boc})\)
A solution of **ABC99_IU** (428.7 mg, 1.87 mmol), *tert*-butyl 2-hydroxy-1,3-dioxohexahydroimidazo[1,5-a]pyrazine-7(1H)-carboxylate (390 mg, 1.44 mmol), triethylamine (1.99 mL, 14.4 mmol), and DMAP (cat.) in THF (12.5 mL) was heated to 70 °C and stirred for 2 hours. After cooling H₂O was added and the product was extracted with ether and concentrated. The resulting residue was purified by flash chromatography (40% EtOAc in hexanes) to yield **preABC99_boc** as a colorless oil (537.4 mg, 87%). ¹H NMR (500 MHz, CDCl₃) δ 7.87 (s, 1H), 7.07 (ddd, J = 8.3, 7.4, 1.5 Hz, 1H), 6.92 (m, 2H), 4.58 (s, 1H), 4.36 (t, J = 4.7 Hz, 2H), 4.10 (m, 5H), 3.03 (m, 3H), 1.49 (s, 9H). HRMS calculated for C₂₀H₂₄N₄O₇ [M+Na]⁺ 455.1537, found 455.1544.

**7-(4-bromobenzyl)-1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-(4-methoxyphenyl)piperazine-1-carboxylate (ABC28)**

**ABC28** was synthesized according to **General Procedure A** using 1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-(4-methoxyphenyl)piperazine1-carboxylate HCl (10.5 mg, 25 µmol) and 4-bromobenzaldehyde (9.3 mg, 50 µmol). Separation by prep-TLC (75% EtOAc in hexanes) afforded **ABC28** (9.4 mg, 68%) as an off-white solid. ¹H NMR (500 MHz, CDCl₃) δ 7.47 (d, J = 8.1 Hz, 2H), 7.20 (d, J = 7.9 Hz, 2H), 6.87 (m, 4H), 4.15 (s, 1H), 4.05 (d, J = 13.3 Hz, 1H), 3.78 (m, 5H), 3.68 (s, 2H), 3.56 (m, 2H), 3.24 (d, J = 10.0 Hz, 1H), 3.12 (br s, 5H), 2.84 (d, J = 11.5 Hz, 1H), 2.15 (s, 2H). HRMS calculated for C₂₅H₂₈BrN₅O₅ [M+H]⁺ 558.1346, found 558.1338.

**7-(4-chlorobenzyl)-1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-(4-methoxyphenyl)piperazine-1-carboxylate (ABC90)**
**ABC90** was synthesized according to **General Procedure A** using 1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-(4-methoxyphenyl)piperazine1-carboxylate (7.2 mg, 16.9 µmol) and 4-chlorobenzaldehyde (9.5 mg, 67.6 µmol). Separation by prep-TLC (75% EtOAc in hexanes) afforded **ABC90** (6.5 mg, 68%) as an off-white solid. $^1$H NMR (500 MHz, CDCl$_3$) δ 7.31 (d, $J = 8.1$ Hz, 2H), 7.25 (m, 2H), 6.89 (m, 4H), 4.15 (s, 1H), 4.05 (d, $J = 13.3$ Hz, 1H), 3.78 (m, 5H), 3.68 (s, 2H), 3.59 (m, 2H), 3.26 (d, $J = 11.8$ Hz, 1H), 3.12 (br s, 5H), 2.85 (d, $J = 11.6$ Hz, 1H), 2.15 (s, 2H). *Note:* peak at 7.25 ppm (2H) overlaps with CHCl$_3$. HRMS calculated for C$_{25}$H$_{28}$ClN$_5$O$_5$ [M+H]$^+$ 514.1852, found 514.1857.

**7-(3-chlorobenzyl)-1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-(4-methoxyphenyl)piperazine-1-carboxylate (ABC91)**

**ABC91** was synthesized according to **General Procedure A** using 1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-(4-methoxyphenyl)piperazine1-carboxylate (7.2 mg, 16.9 µmol) and 3-chlorobenzaldehyde (9.5 mg, 67.6 µmol). Separation by prep-TLC (75% EtOAc in hexanes) afforded **ABC91** (6.5 mg, 68%) as an off-white solid. $^1$H NMR (500 MHz, CDCl$_3$) δ 7.34 (s, 1H), 7.27 (d, $J = 4.6$ Hz, 2H), 7.19 (s, 1H), 6.89 (m, 4H), 4.19 (s, 1H), 4.06 (d, $J = 13.4$ Hz, 1H), 3.78 (m, 6H), 3.59 (m, 3H), 3.26 (br s, 1H), 3.14 (br s 4H), 3.02 (br s, 1H), 2.87 (br s, 1H), 2.18 (m, 2H). HRMS calculated for C$_{25}$H$_{28}$ClN$_5$O$_5$ [M+H]$^+$ 514.1852, found 514.1853.

**7-(3-chlorobenzyl)-1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-(4-methoxyphenyl)piperazine-1-carboxylate (ABC92)**
**ABC92** was synthesized according to **General Procedure A** using 1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-(4-methoxyphenyl)piperazine-1-carboxylate (7.2 mg, 16.9 µmol) and 2-chlorobenzaldehyde (9.5 mg, 67.6 µmol). Separation by prep-TLC (75% EtOAc in hexanes) afforded **ABC92** (4.8 mg, 51%) as an off-white solid. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 7.39 (m, 2H), 7.26 (m, 1H), 6.89 (m, 4H), 4.18 (s, 1H), 4.06 (d, \(J = 13.5\) Hz, 1H), 3.78 (m, 9H), 3.33 (s, 1H), 3.14 (s, 4H), 3.02 (s, 1H), 2.91 (s, 1H), 2.33 (m, 2H). *Note:* peak at 7.26 ppm (1H) overlaps with CHCl\(_3\). HRMS calculated for C\(_{25}\)H\(_{28}\)ClN\(_5\)O\(_5\) [M+H]\(^+\) 514.1852, found 514.1854.

1,3-dioxo-7-((6-phenoxypyridin-3-yl)methyl)hexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 2,3-dihydro-4\(H\)-benzo[b][1,4]oxazine-4-carboxylate (ABC63)

**ABC63** was synthesized according to **General Procedure A** using 6-phenoxynicotinaldehyde (19.1 mg, 96 µmol) and 1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 2,3-dihydro-4\(H\)-benzo[b][1,4]oxazine-4-carboxylate (derived from 7-(tert-butoxycarbonyl)-1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 2,3-dihydro-4\(H\)-benzo[b][1,4]oxazine-4-carboxylate using **General Procedure B**) (8.1 mg, 24 µmol). Separation by prep-TLC (75% EtOAc in hexanes) afforded **ABC63** (3.3 mg, 26%) as an off-white solid. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 8.10 (d, \(J = 2.4\) Hz, 1H), 7.87 (s, 1H), 7.71 (s, 1H), 7.41 (m, 2H), 7.22 (m, 1H), 7.16 (m, 2H), 7.06 (m, 1H), 6.91 (m, 3H), 4.36 (t, \(J = 4.7\) Hz, 2H), 4.08 (m, 4H), 3.55 (br s, 2H), 3.29 (m, 2H), 2.92 (s, 1H), 2.31 (m, 2H). HRMS calculated for C\(_{27}\)H\(_{25}\)N\(_3\)O\(_6\) [M+H]\(^+\) 516.1878, found 516.1882.

7-(4-chlorobenzyl)-1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 2,3-dihydro-4\(H\)-benzo[b][1,4]oxazine-4-carboxylate (ABC99)
ABC99 was synthesized according to General Procedure A using 4-chlorobenzaldehyde (123 mg, 658 µmol) and 1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 2,3-dihydro-4H-benzo[b][1,4]oxazine-4-carboxylate (derived from 7-(tert-butoxycarbonyl)-1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 2,3-dihydro-4H-benzo[b][1,4]oxazine-4-carboxylate using General Procedure B) (80.4 mg, 218 µmol). Separation by prep-TLC (75% EtOAc in hexanes) afforded ABC99 (73.8 mg, 74%) as a white solid. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.88 (s, 1H), 7.31 (m, 2H), 7.25 (m, 2H), 7.06 (m, 1H), 6.91 (m, 2H), 4.36 (t, $J$ = 4.7 Hz, 2H), 4.17 (dd, $J$ = 11.1, 4.3 Hz, 1H), 4.06 (m, 3H), 3.58 (m, 2H), 3.25 (ddd, $J$ = 11.3, 4.5, 1.6 Hz, 1H), 3.15 (ddd, $J$ = 13.2, 11.9, 3.8 Hz, 1H), 2.85 (ddt, $J$ = 11.6, 3.6, 1.5 Hz, 1H), 2.17 (br s, 2H). HRMS calculated for C$_{22}$H$_{21}$ClN$_4$O$_5$ [M+H]$^+$ 457.1273, found 457.1282.

7-(4-chlorobenzoyl)-1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 2,3-dihydro-4H-benzo[b][1,4]oxazine-4-carboxylate (ABC101)
To a stirring solution of 1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 2,3-dihydro-4H-benzo[b][1,4]oxazine-4-carboxylate (derived from 7-(tert-butoxycarbonyl)-1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 2,3-dihydro-4H-benzo[b][1,4]oxazine-4-carboxylate using **General Procedure B**) (53.3 mg, 161 µmol) and triethylamine (67 µl, 482 µmol) in DCM (1.6 mL), 4-chlorobenzoyl chloride (36.5 mg, 209 µmol) was added. The resulting solution was heated to 45 °C and stirred for two hours, after which the mixture was allowed to cool to room temperature and the solvent was removed under nitrogen. Separation of the resulting residue by prep-TLC (75% EtOAc in hexanes) afforded **ABC101** (70.4 mg, 93%).

**1H NMR** (500 MHz, CDCl₃) δ 7.86 (s, 1H), 7.45 (m, 2H), 7.39 (m, 2H), 7.07 (td, \(J = 7.7, 1.5\) Hz, 1H), 6.92 (m, 2H), 4.29 (m, 8H), 3.11 (br s, 3H). HRMS calculated for C₂₂H₁₉ClN₄O₆ [M+H]+ 471.1066, found 471.1066.

**Synthesis of ABC99yne**

**tert-butyl 4-(prop-2-yn-1-yl)-3,4-dihydroquinoxaline-1(2H)-carboxylate**

(ABC99yne_SG_boc)

A stirring solution of **tert**-butyl 3,4-dihydro-2H-quinoxaline-1-carboxylate (Combi-Blocks) (200 mg, 854 µmol), propargyl bromide (203 mg, 1708 µmol, 80%/wt in toluene), sodium iodide (128 mg, 854 µmol), and potassium carbonate (236 mg, 1708 µmol) in DMF (2 mL) was heated to 50 °C for four hours. The solution was then washed with brine (2x), concentrated, and the resulting residue was purified by prep-TLC (10% EtOAc in hexanes) to yield **ABC99yne_SG_boc** (144 mg, 62%) as a yellow oil. **1H NMR** (500 MHz, CDCl₃) δ 7.52 (d, \(J = 7.9\) Hz, 1H), 7.02 (dd, \(J = 8.2, 7.3, 1.5\) Hz, 1H), 6.80 (dd, \(J = 8.2, 1.4\) Hz, 1H), 6.73 (ddd, \(J = 8.1, 7.3, 1.4\) Hz, 1H), 4.04 (d,
$J = 2.3$ Hz, 2H), 3.83 (m, 2H), 3.41 (m, 2H), 2.19 (t, $J = 2.4$ Hz, 1H), 1.52 (s, 9H). HRMS calculated for $\text{C}_{16}\text{H}_{20}\text{N}_{2}\text{O}_{2} \ [\text{M+H}]^+$ 273.1597, found 273.1597.

(1H-imidazol-1-yl)(4-(prop-2-yn-1-yl)-3,4-dihydroquinoxalin-1(2H)-yl)methanone (ABC99yne_IU)

To a stirring solution of carbonyldiimidazole (49.4 mg, 305 µmol) in DCM (0.5 mL) 1-(prop-2-yn-1-yl)-1,2,3,4-tetrahydroquinoxaline (derived from ABC99yne_SG_boc using General Procedure B) (35 mg, 203 µmol) was added. The resulting solution was stirred overnight at room temperature, then washed with H$_2$O, concentrated, and purified by prep-TLC (EtOAc) to yield ABC99yne_IU (46.6 mg, 86%) as a yellow oil. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.74 (t, $J = 1.0$ Hz, 1H), 7.11 (ddd, $J = 8.2$, 7.1, 1.7 Hz, 1H), 7.03 (t, $J = 1.4$ Hz, 1H), 6.93 (m, 2H), 6.58 (m, 2H), 4.11 (d, $J = 2.4$ Hz, 2H), 4.04 (t, $J = 5.7$ Hz, 2H), 3.60 (t, $J = 5.7$ Hz, 2H), 2.24 (t, $J = 2.4$ Hz, 1H). HRMS calculated for $\text{C}_{15}\text{H}_{14}\text{N}_{4}\text{O} \ [\text{M+H}]^+$ 267.1240, found 267.1247.

7-(tert-butoxycarbonyl)-1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-(prop-2-yn-1-yl)-3,4-dihydroquinoxaline-1(2H)-carboxylate (preABC99yne_boc)

A stirring solution of ABC99yne_IU (31 mg, 116 µmol), tert-butyl 2-hydroxy-1,3-dioxohexahydroimidazo[1,5-a]pyrazine-7(1H)-carboxylate (41 mg, 151 µmol), triethylamine (161 µL, 1164 µmol) and 4-dimethylaminopyridine (cat.) in THF was heated to 70 °C and stirred for two hours. The solution was then concentrated, and the resulting residue was purified by prep-TLC (75% EtOAc in hexanes) to yield preABC99yne_boc (46.9 mg, 86%) as a yellow solid. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.63 (d, $J = 8.0$ Hz, 1H), 7.10 (ddd, $J = 8.6$, 7.4, 1.5 Hz, 1H), 6.83 (dd, $J = 8.4$, 1.3 Hz, 1H), 6.76 (ddd, $J = 8.5$, 7.4, 1.3 Hz, 1H), 4.57 (s, 1H), 4.07 (m,
7H), 3.53 (t, $J = 5.4$ Hz, 2H), 3.01 (m, 3H), 2.22 (t, $J = 2.4$ Hz, 1H), 1.49 (s, 9H). HRMS calculated for $C_{23}H_{27}N_5O_6$ [M+Na]$^+$ 492.1853, found 492.1855.

7-(4-chlorobenzyl)-1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-(prop-2-yn-1-yl)-3,4-dihydroquinoxaline-1(2H)-carboxylate (ABC99yne)

ABC99yne was synthesized according to General Procedure A using 4-chlorobenzaldehyde (18.9 mg, 67.2 µmol) and 1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-(prop-2-yn-1-yl)-3,4-dihydroquinoxaline-1(2H)-carboxylate (derived from preABC99yne_boc using General Procedure B) (12.4 mg, 33.6 µmol). Separation by prep-TLC (50% EtOAc in hexanes) afforded ABC99yne (15.1 mg, 91%) as a yellow solid. $^1$H NMR (500 MHz, CDCl$_3$) δ 7.63 (d, $J = 7.8$ Hz, 1H), 7.31 (d, $J = 8.3$ Hz, 2H), 7.25 (m, 2H), 7.09 (m, 1H), 6.83 (d, $J = 8.4$ Hz, 1H), 6.76 (m, 1H), 4.16 (m, 1H), 4.04 (m, 5H), 3.55 (m, 4H), 3.24 (dd, $J = 11.3$, 4.5 Hz, 1H), 3.13 (m, 1H), 2.84 (m, 1H), 2.22 (m, 3H). HRMS calculated for $C_{25}H_{24}ClN_5O_4$ [M+H]$^+$ 494.1589, found 494.1592.
References

1. Cognetta, A. B., 3rd; Niphakis, M. J.; Lee, H. C.; Martini, M. L.; Hulce, J. J.; Cravatt, B. F., Selective N-Hydroxyhydantoin Carbamate Inhibitors of Mammalian Serine Hydrolases. *Chem Biol* **2015**, 22 (7), 928-37.

2. Adibekian, A.; Martin, B. R.; Wang, C.; Hsu, K. L.; Bachovchin, D. A.; Niessen, S.; Hoover, H.; Cravatt, B. F., Click-generated triazole ureas as ultrapotent in vivo-active serine hydrolase inhibitors. *Nat Chem Biol* **2011**, 7 (7), 469-78.

3. Hsu, K. L.; Tsuboi, K.; Adibekian, A.; Pugh, H.; Masuda, K.; Cravatt, B. F., DAGLbeta inhibition perturbs a lipid network involved in macrophage inflammatory responses. *Nat Chem Biol* **2012**, 8 (12), 1000-7.

4. Hsu, K. L.; Tsuboi, K.; Chang, J. W.; Whitby, L. R.; Speers, A. E.; Pugh, H.; Cravatt, B. F., Discovery and optimization of piperidyl-1,2,3-triazole ureas as potent, selective, and in vivo-active inhibitors of alpha/beta-hydrolase domain containing 6 (ABHD6). *J Med Chem* **2013**, 56 (21), 8270-9.

5. Inloes, J. M.; Hsu, K. L.; Dix, M. M.; Viader, A.; Masuda, K.; Takei, T.; Wood, M. R.; Cravatt, B. F., The hereditary spastic paraplegia-related enzyme DDHD2 is a principal brain triglyceride lipase. *Proc Natl Acad Sci U S A* **2014**, 111 (41), 14924-9.

6. Chang, J. W.; Niphakis, M. J.; Cognetta, A. B., 3rd; Wang, C.; Matthews, M. L.; Niessen, S.; Buczynski, M. W.; Parsons, L. H.; Cravatt, B. F., Highly selective inhibitors of monoacylglycerol lipase bearing a reactive group that is bioisosteric with endocannabinoid substrates. *Chem Biol* **2012**, 19 (5), 579-88.

7. Weerapana, E.; Wang, C.; Simon, G. M.; Richter, F.; Khare, S.; Dillon, M. B.; Bachovchin, D. A.; Mowen, K.; Baker, D.; Cravatt, B. F., Quantitative reactivity profiling predicts functional cysteines in proteomes. *Nature* **2010**, 468 (7325), 790-5.

8. Xu, T.; Park, S. K.; Venable, J. D.; Wohlschlegel, J. A.; Diedrich, J. K.; Cociorva, D.; Lu, B.; Liao, L.; Hewel, J.; Han, X.; Wong, C. C. L.; Fonslow, B.; Delahunty, C.; Gao, Y.; Shah, H.; Yates, J. R., 3rd, ProLuCID: An improved SEQUEST-like algorithm with enhanced sensitivity and specificity. *J Proteomics* **2015**, 129, 16-24.

9. Cociorva, D.; L. T.; Yates, J. R., Validation of tandem mass spectrometry database search results using DTASelect. *Curr Protoc Bioinformatics* **2007**, Chapter 13, Unit 13 4.

10. Xu, Q.; Wang, Y.; Dabdoub, A.; Smallwood, P. M.; Williams, J.; Woods, C.; Kelley, M. W.; Jiang, L.; Tasman, W.; Zhang, K.; Nathans, J., Vascular development in the retina and inner ear: Control by Norrin and Frizzled-4, a high-affinity ligand-receptor pair. *Cell* **2004**, 116 (6), 883-895.