Supporting Information for:

Covalent Modulators of the Vacuolar ATPase

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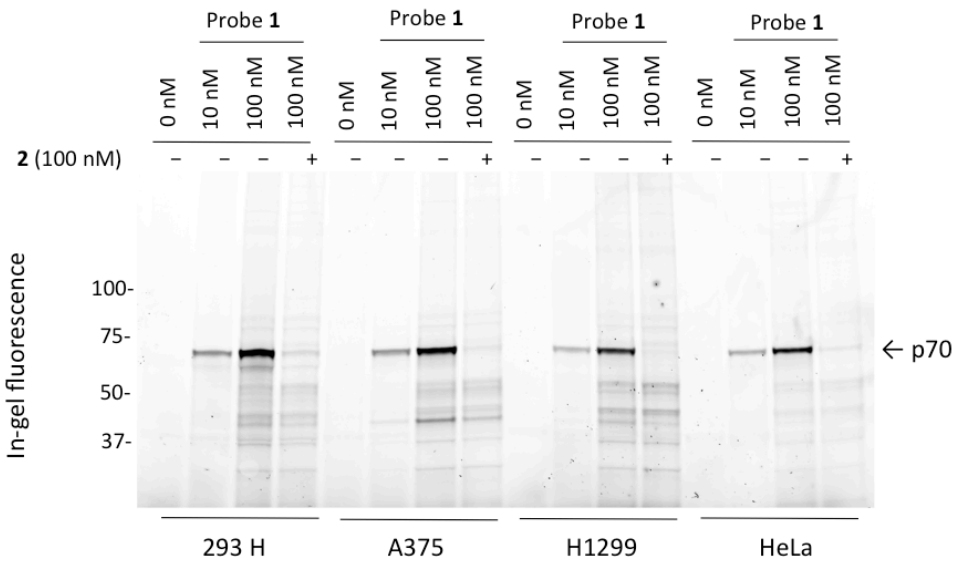


Figure S1. Specific labeling of p70 by probe **1** in a number of human cancer cell lines. Cells were pretreated with DMSO or compound **2** for 20 min at 37 °C, and incubated with probe **1** at indicated concentrations for 20 min at 37 °C. Cells were then lysed, subjected to CuACC-mediated conjugation with TAMRA-azide, resolved by SDS-PAGE, and scanned for fluorescence.

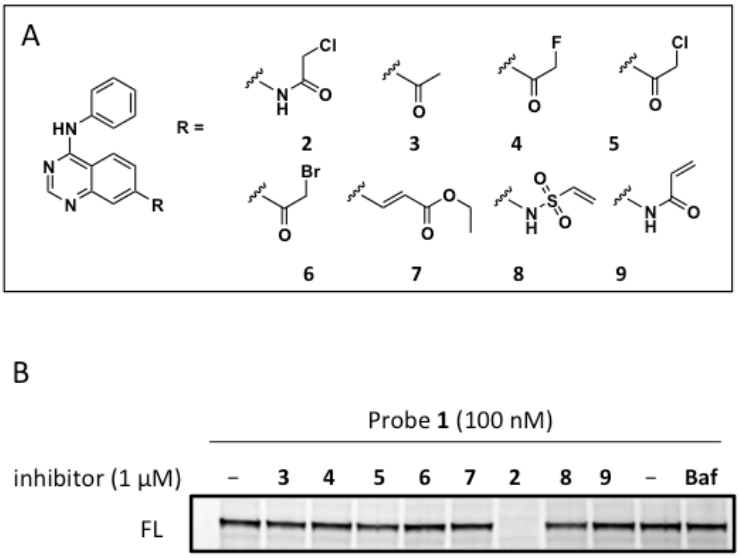


Figure S2. Screening of a panel of electrophilic quinazolines for occupancy of the p70 protein target. (A) Structures of quinazolines **2** – **8**. (B) Screening of the quinazolines revealed that only the chloroacetamide **2** blocked probe **1** labeling of the p70 protein. 293 cells were pretreated with DMSO, a quinazoline (**2**-**8**), or bafilomycin (Baf) for 30 min at 37 °C, and incubated with 100 nM of probe **1** for 30 min at 37 °C. Cells were then lysed, subjected to CuACC-mediated conjugation with TAMRA-azide, resolved by SDS-PAGE, and scanned for fluorescence (FL).

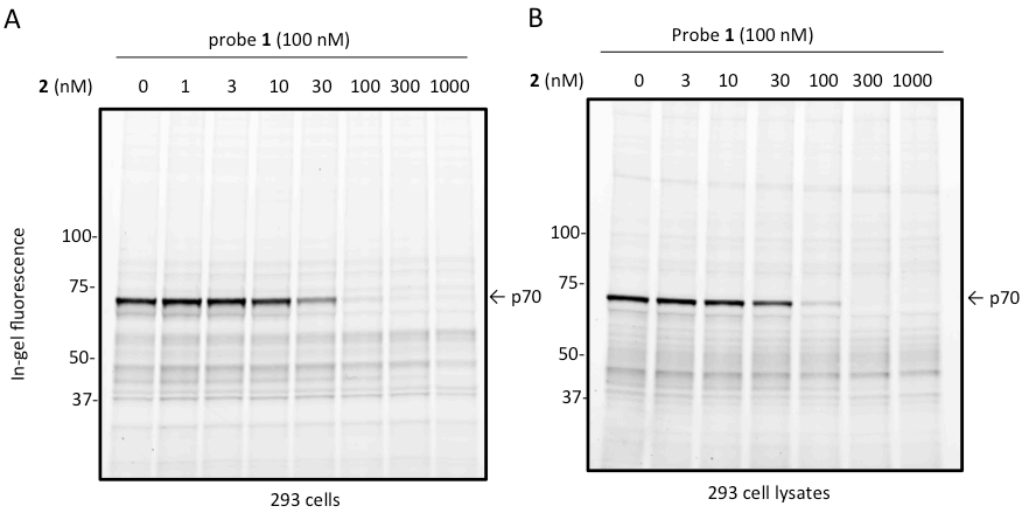


Figure S3. Competition experiments revealed similar potency of compound **2** at occupying the p70 protein both *in vitro* and *in situ*. 293 live cells (A) or cell lysates (B) were pretreated with various concentrations of compound **2** for 20 min at 37 °C before incubation with 100 nM of probe **1** at 37 °C for another 20 min. Cells were then lysed, conjugated to TAMRA-azide via CuAAC, resolved by SDS-PAGE, and scanned for fluorescence.

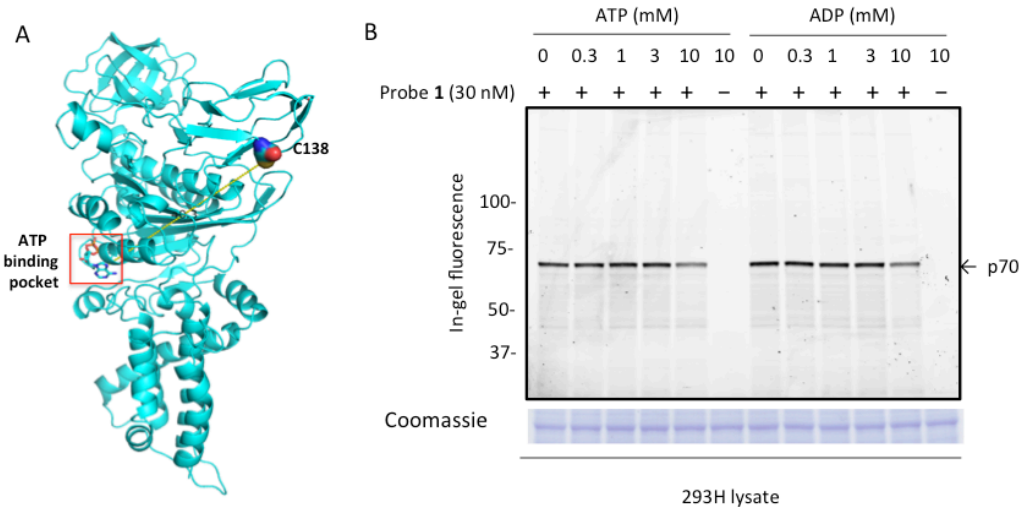


Figure S4. ATP and ADP failed to compete off p70 protein labeling by probe **1**. (A) A homology model of human ATP6V1A reveals that C138 is approximately 40 Å away from the ATP binding pocket in the model. The homology model was generated with SWISS-MODEL (https://swissmodel.expasy.org) using the EM structure of the yeast protein as the template.[1] (B) ATP and ADP had little effect on p70 protein labeling by probe **1**. 293 cell lysates were cotreated with various concentrations of ATP or ADP and probe **1** (30 nM) at 37 °C for 20 min. Cells were then lysed, conjugated to TAMRA-azide via CuAAC, resolved by SDS-PAGE, and scanned for fluorescence. Coomassie staining was used as a loading control.

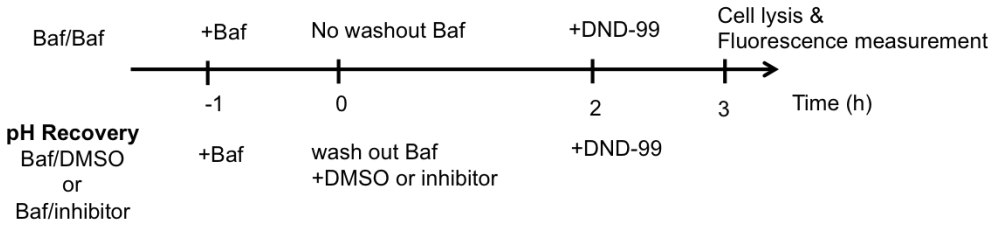


Figure S5. A flowchart illustrating the procedure for the intracellular vesicle re-acidification assay. Cells were treated with bafilomycin (Baf) for 1 h before being washed or not. DMSO or an inhibitor was then added for 2 h before DND99 was added for additional 1 h. Fluorescence was measured at the end. Continuous Baf treatment (Baf/Baf) and recovery in the presence of DMSO (Baf/DMSO) served respectively as a positive control and negative control in the re-acidification inhibition assay.

MATERIALS & METHODS

Cell culture

Cells (obtained from ATCC or ThermoFisher Scientific) were grown at 37 ^{o}C under a humidified 5 % CO_2 atmosphere with proper growth media. 4.5 g/L glucose DMEM (Corning) supplemented with 10% FBS and L-glutamine was used for HEK293, A375 and HeLa cells; RPMI supplemented with 10% FBS and L-glutamine for H1299; DMEM supplemented with 10% FCS, Pen Strep and L-glutamine for MDA-MB-231.

Probe treatment *in situ* and in-gel fluorescence imaging

Cells were grown to ~90% confluence in 6-well plates with proper media with 10% FBS. The growth media was removed and the cells were treated with fresh media containing various concentrations of probe (1,000× stock solution in DMSO) or vehicle control for the indicated time. For the IC_{50} measurements, cells were first incubated with compound **2** at various concentrations for 20 min at 37 ^{o}C and then treated with 100 nM probe **1** for another 20 min at 37 ^{o}C. After the probe treatment, the media was removed and the cells were washed twice with ice-cold DPBS. The cells were harvested and the pellet was resuspended in 80 μL of NP40 lysis buffer (50 mM HEPES, pH 7.4, 1% NP-40, 150 mM NaCl) with protease inhibitor cocktail (Roche). The lysate was incubated on ice for 20 min and fractionated by centrifugation at 18,000 × *g* for 10 min at 4 ^{o}C. The protein concentrations were measured from each of the supernatant sample by BCA assay (Pierce) and adjusted to 1 mg/mL. CuACC was performed at a final concentration of 25 μM TAMRA-azide (Click Chemistry Tools), 1 mM Tris(2-carboxyethyl)phosphine (TCEP, Thermo-Scientific), 100 μM Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA, TCI), and 1 mM $CuSO_4$ (Sigma-Aldrich) in a total volume of 100 μL. The reaction was performed at RT for 1 h in the dark before termination by addition of 40 μL of 4× Laemmli sample buffer (Bio-Rad) and boiled for 5 min. 30 μL of the samples were loaded and resolved on a 4-20% SDS-PAGE before visualization at 532 nm for excitation and 610 nm for emission on a Typhoon 9400 Variable Mode Imager (GE Healthcare). Fluorescence images are displayed as gray scale. For the cells transfected with 3×FLAG tagged V-ATPase, after fluorescence scanning, proteins were transferred to PVDF membranes for immunoblotting with FLAG antibodies to monitor the transfection level.

Probe treatment *in vitro* and in-gel fluorescence imaging

Untreated 293 cells were harvested and lysed as described above, and the proteome concentrations were adjusted to 1 mg/mL concentration in a volume of 98 μL. For competition experiments with compound **2** (1,000× stock solution in DMSO) or 3-BP (freshly prepared 10× stock solution in 100 mM Hepes buffer, pH 7.3), lysates were first incubated with various concentrations of the compound for 20 min at 37 ^{o}C, followed by addition of probe **1** (100 nM) for another 20 min at 37 ^{o}C. Click reactions were set up in the dark for 1 h (the concentration of click reagents are described above). The samples were treated with 40 μL of 4× Laemmli sample buffer (Bio-Rad) and boiled for 5 min. 30 μL of the samples were loaded and resolved on a 4-20% SDS-PAGE and imaged as described above. For competition experiments with nucleotides, 10 mM of $MgCl_2$ and various concentrations of ATP (or ADP) were added to 1 mg/mL proteome from 293 cells in NP40 buffer in a total volume of 49 μL. Lysates were incubated with probe **1** (30

nM) for another 20 min at 37 ^{o}C. Click reactions were set up in the dark for 0.5 h (the concentrations of click reagents are described above). The samples were treated with 20 μL of 4× Laemmli sample buffer (Bio-Rad) and boiled for 5 min. 30 μL of the samples were loaded and resolved on a 4-20% SDS-PAGE gel and imaged as described above.

Biotin-streptavidin enrichment and protein identification

293 cells were grown to 90% confluence in three 15 cm plates and incubated with DMSO or probe **1** (100 nM) for 30 min at 37 ^{o}C. Cells were harvested as described above and then resuspended in lysis buffer (50 mM HEPES, pH = 7.4, 150 mM NaCl, 1% NP40, 7 mM PMSF, with Roche protease inhibitor cocktail). Cells were briefly sonicated in a bath sonicator and incubated on ice for another 20 min before centrifugation at 20,000 × *g* at 4 ^{o}C for 10 min. Protein concentrations of cell lysates were adjusted to 1 mg/mL (total 10 mg of protein) and the lysates were subjected to CuACC with 50 μM biotin-azo-azide, (click chemistry tool, cat. no. 1041), 1 mM TCEP, 100 μM TBTA, and 1mM Cu_2SO_4 in a total volume of 10 mL and incubated in the dark for 1 h. 4× reaction volume of ice-cold MeOH were added to the reaction mixtures and the proteins were allowed to precipitate at -20 ^{o}C overnight. Precipitated proteins were collected by centrifugation at 5,200 × *g* for 30 min and the pellets were washed twice with ice-cold MeOH. The pellets were then air-dried and resuspended in HEPES buffer (6 M Urea, 2 M thiourea, 10 mM HEPES, pH = 8.0), and supplemented with 1 mM DTT and incubated at RT for 40 min, followed by 5.5 mM iodoacetamide and incubated at RT for a further 30 min in the dark. The resulting cell lysates were incubated with streptavidin beads for 2 h at RT and the beads were further washed with PBS and 1% SDS PBS buffer. Lastly, the beads were incubated with 50 mM sodium dithionite to release the captured biotinylated proteins. The captured proteins were resolved on SDS-PAGE gel, excised and analyzed at the Proteomic Resource Center, The Rockefeller University (New York). More details can be found in our previous publication.[2]

Plasmid construction and transfection

All constructs were prepared using standard molecular cloning techniques unless obtained from other labs as indicated below. Human and mouse ATP6V1A coding regions were amplified by PCR and cloned into p3×FLAG-CMV10 vector. All mutations were generated using QuikChange™ Lightning Site-directed Mutagenesis (Agilent). Detailed cloning information and sequences will be made available upon request. For transfection, 293H cells were seeded at $5x10^5$ cells per well in 6-well plates 16 hours before transfection with V-ATPase constructs (p3xFLAG-CMV10) using Lipofectamine 2000 (Life Technologies). The cells were analyzed 24 h after transfection.

Western blotting

After SDS-PAGE gel, the proteins were transferred to a PVDF membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). The membrane was incubated with antibody according to manufacturer's instruction and developed using ECL substrate (Bio-Rad) and imaged with ChemiDoc XRS+ molecular imager (Bio-Rad). The following antibodies were used: anti-Flag tag (Sigma, cat# F3165, 1:8000 dilution) and anti-ATP6V1A (house-made,[3] 1:2000 dilution).

isoTOP-ABPP analysis

MDA-MB-231 cells (HTB. 26™), obtained from ATCC and tested negative for mycoplasma, were cultured at 37 ºC with 5% CO_2 in DMEM medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and glutamine. Cells were maintained at a low passage number (<10 passages). Cells were harvested by centrifugation (1,400 × *g*, 3 min, 4 °C); pellets were washed with cold PBS, lysed by sonication and fractionated (100,000 × *g*, 45 min) to yield the soluble fraction, which was then adjusted to a final protein concentration of 1.5 mg/mL. Protein concentration was determined using the Bio-Rad DC™ protein assay kit. 500 µL of the indicated proteome was treated with compound **2** (5 µL of 50 µM DMSO solution, final concentration = 500 nM) or with vehicle (5 µL DMSO). Samples were incubated for 1 h, after which treated and control samples were further labeled for an additional 1 h with iodoacetamide-alkyne (IA-alkyne, 5 µL of 10 mM stock in DMSO, final concentration = 100 µM). All labeling steps were conducted at RT. Control and treated samples were then subjected to copper-mediated azide-alkyne cycloaddition (CuAAC) conjugation to isotopically-labeled, TEV-cleavable biotinylated peptide tags. Control and treated samples were subsequently combined, enriched on streptavidin resin (Pierce 20349), and subjected to sequential trypsin and TEV digests as reported previously.[4] TEV digests were analyzed by multidimensional liquid chromatography tandem mass spectrometry (MudPIT) using an LTQ-Velos Orbitrap mass spectrometer (Thermo Scientific) coupled to an Agilent 1200- series quaternary pump and searched and analyzed as has been reported previously.[4]

Quantitative fluorescence-based vesicle acidification assay

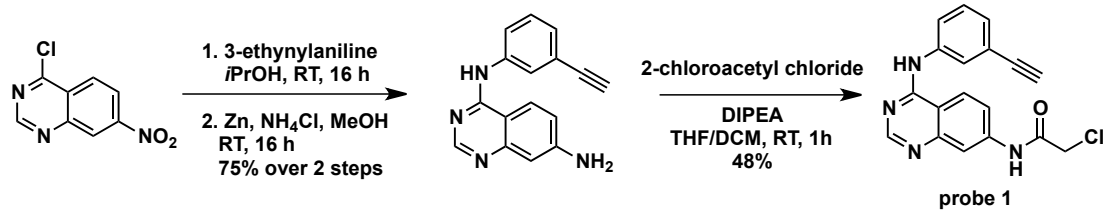
HeLa (human cervix epithelium) cells were seeded in 24-well cell culture plates on the day before the experiment. For assay under standard growth conditions, wells in duplicates were either treated with 0.1% DMSO (vehicle-only control), 10 µM concentrations of compounds **1** or **2** (in 0.1% DMSO), or 100 nM bafilomycin A1 (Sigma-Aldrich, St. Louis, MO), which was used as a positive control of inhibition of intravesicular acidification. After 1 h from the start of treatment, LysoTracker Red DND-99 (Thermo Fisher Scientific, Waltham, MA) was added to a final concentration of 1 µM for another 1 h (thus, total time of treatment with compounds was 2 h). After labeling with DND-99, cells in all wells were rinsed twice in PBS and lysed in 200 µL of RIPA lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS); the lysate was then transferred into a black 96-well plate (Corning, Corning, NY). Fluorescence released into the medium was read immediately using a Multimode Detector plate reader (model DTX 880; Beckman-Coulter, Fullerton, CA) with the following settings: fluorescence intensity top method, 0.4-s integration time, 535-nm excitation filter, and 595-nm emission filter. Fluorescence values were then normalized to vehicle-only control value, which was taken as 100%. All assays were done in duplicates and repeated at least 3 times for each compound with similar results. Representative experiments are shown for each compound. Graphs showing mean ± standard deviation of the mean were plotted with Microsoft Excel for Mac 2011 software (Microsoft, Redmond, WA).

For assay using cells pre-treated with bafilomycin A1, two wells (for duplicates) were treated with 0.1% DMSO (vehicle-only control) and two other wells were treated with

100 nM bafilomycin A1 (positive control of inhibition) for the total duration of the assay (4 h), while all other wells were first pre-incubated with 100 nM bafilomycin A1 for 1 h, which was then washed out and either 0.1% DMSO or compound **1** was immediately added to the final concentrations of 1 nM, 10 nM, 1 μM or 10 μM. 2 h later, cells were labeled with 1 μM LysoTracker Red DND-99 for 1 h to follow the recovery of intracellular vesicle acidification after bafilomycin washout. Cells were lysed, fluorescence was measured, and data analysis was done as described above for the assay under standard growth conditions. IC_{50} of the inhibition of re-acidification by compound **1** was calculated using nonlinear regression "Dose-Response – Inhibition" model built-in to Prism 7 software (GraphPad Software, La Jolla, CA). Data was first normalized to re-acidification in the presence of 0.1% DMSO values taken as 100%, and positive control of inhibition (100 nM Bafilomycin A1, 4 h) values taken as 0%. Graphs showing mean ± standard deviation of the mean were plotted with Microsoft Excel for Mac 2011 software (Microsoft, Redmond, WA).

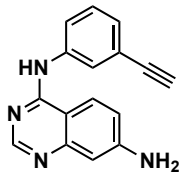
Synthetic Methods

Unless noted otherwise, all chemicals and reagents were obtained via commercial sources and were used without further purification. Flash chromatography was carried out using an automated system (Teledyne Isco CombiFlash) or manually with 230–400 mesh silica gel. Mass spectrometry data were collected on an Agilent 6100 Series Quadrupole LC/MS or on Thermo Scientific™ Exactive Mass Spectrometer with DART ID-CUBE for high-resolution mass spectrometry (HR-MS). All anhydrous reactions were carried out under nitrogen atmosphere. NMR spectra were obtained on Varian VNMRS-500 or Mercury-400. Multiplicities are reported using the following abbreviations: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad). Synthesis of compound **2** – **9** has been described in our previous publication.[5]



Scheme 1. Synthesis of probe **1**

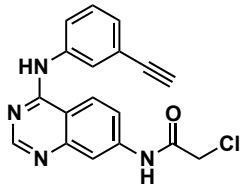
***N*[4]-(3-ethynylphenyl)quinazoline-4,7-diamine**



To a solution of 4-chloro-7-nitroquinazoline[5] (75 mg, 0.36 mmol) in isopropanol (2 mL) was added 3-ethynylaniline (43 mg, 0.37 mmol). The reaction mixture was stirred at RT for 12 h and then cooled in an ice bath. The precipitate was collected through filtration, washed with cold isopropanol/hexane, and dried over vacuo to afford *N*-(3-ethynylphenyl)-7-nitroquinazolin-4-amine as a yellow solid (100 mg). The residues were further suspended in methanol (2 mL), and NH_4Cl (98 mg, 1.8 mmol) and Zn powder (100 mg, 1.5 mmol) were added. The reaction mixture was sonicated in a bath sonicator for 5 min and stirred at RT for 16 h. The reaction mixture was filtered to remove the residual zinc powder, which was then concentrated in vacuo. The residues were diluted with EtOAc and washed with saturated $NaHCO_3$ solution. The basic layer was further extracted with EtOAc twice. The combined organic phases were washed with brine, dried over $MgSO_4$(s), and concentrated under reduced pressure to provide the desired compound as a yellow solid (70 mg, 75% yield over 2 steps).
^{1}H NMR (500 MHz, dmso) δ 9.37 (s, 1H), 8.38 (s, 1H), 8.18 (d, J = 9.0 Hz, 1H), 8.05 (t, J = 2.0 Hz, 1H), 7.84–7.92 (m, 1H), 7.35 (t, J = 8.0 Hz, 1H), 7.12–7.20 (m, 1H), 6.91 (dd, J = 9.0, 2.5 Hz, 1H), 6.71 (d, J = 2.5 Hz, 1H), 6.06 (s, 2H), 4.16 (s, 1H); ^{13}C NMR (125 MHz, dmso) δ 156.83, 154.33, 153.01, 151.99, 140.17, 128.76, 125.82, 124.22, 123.88,

122.05, 121.61, 116.67, 105.90, 105.67, 83.68, 80.35. **ESI-MS** calculated for $C_{16}H_{12}N_4$ $[M+H]^+$ m/z 261.1, found 261.2.

2-chloro-*N*-(4-((3-ethynylphenyl)amino)quinazolin-7-yl)acetamide



To a solution of N^4-(3-ethynylphenyl)quinazoline-4,7-diamine (50 mg, 0.19 mmol) in THF/CH_2Cl_2 (5:1, 2.0 mL) DIPEA (100 μL, 0.58 mmol) was added and stirred for 10 min. Chloroacetylchloride (23 μL, 0.29 mmol) was added dropwise to the reaction mixture and stirred for another 1 h, at which time TLC indicated complete conversion. The mixture was quenched with water, diluted with EtOAc, and washed with saturated $NaHCO_3$ solution. The aqueous phase was extracted with EtOAc twice more and the combined organic phases were dried over anhydrous $MgSO_{4(s)}$, concentrated in vacuo, and purified by silica gel chromatography (1–2% MeOH gradient in CH_2Cl_2) to afford the desired compound as a cream solid (31 mg, 48%).
^{1}H NMR (500 MHz, dmso) δ 10.81 (s, 1H), 9.77 (s, 1H), 8.60 (s, 1H), 8.50 (d, J = 9.5 Hz, 1H), 8.14 (d, J = 2.5 Hz, 1H), 8.07 (t, J = 2.0 Hz, 1H), 7.88–7.94 (m, 1H), 7.75 (dd, J = 9.5, 2.5 Hz, 1H), 7.40 (t, J = 8.0 Hz, 1H), 7.20–7.25 (m, 1H), 4.36 (s, 2H), 4.20 (s, 1H); ^{13}C NMR (125 MHz, dmso) δ 165.42, 157.18, 154.94, 150.68, 142.41, 139.50, 128.92, 126.66, 124.81, 124.02, 122.62, 121.75, 118.87, 115.15, 111.32, 83.49, 80.61, 43.64. **HR-MS** calculated for $C_{18}H_{13}ClN_4O$ $[M–H]^+$ m/z 335.06996, found 335.06923.

Reference:

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