Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Cellular Thermal Shift and Clickable Chemical Probe Assays for the Determination of Drug-Target Engagement in Live Cells

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Synthesis of Atto-DAQ

The 5-(piperidin-4-ylmethoxy)-quinazoline-2,4-diamine[1] (0.6 mg, 0.002 mmol), was combined with the Atto 488 NHS ester purchased from Sigma-Aldrich (2 mg, 0.002 mmol), diluted with 0.4 mL of dry *N*,*N*-dimethylformamide (DMF), and treated with 1 drop of *N*,*N*-diisopropylethylamine (DIEA). The vial was capped, wrapped in foil, and stirred at ambient temperature overnight. The reaction solution was then purified by reverse phase directly on a Waters Sunfire C18 19x100, 5μ column with acetonitrile/water each containing 0.05% trifluoroacetic acid (TFA), to give the desired material as a bright pink solid (1.2 mg, 70% yield). ^{1}H NMR (500 MHz, DMSO) δ 9.20 (br. s., 1H), 8.48 (br. s., 1H), 8.30-8.42 (m, 1H), 8.08-8.20 (m, 1H), 7.66-7.76 (m, 2H), 7.63 (m, 1H), 7.38-7.48 (m, 1H), 7.07 (m, 2H), 6.99 (m, 2H), 6.79-6.87 (m, 1H), 4.27-4.43 (m, 1H), 4.11-4.17 (m, 1H), 3.90-4.04 (m, 2H), 3.38-3.47 (m, 1H), 3.11-3.27 (m, 2H), 2.93 (m, 2H), 2.66 (m, 1H), 2.55 (s, 1H), 2.21 (br. s., 1H), 1.86-1.98 (m, 1H), 1.72-1.85 (m, 1H), 1.59-1.69 (m, 1H), 1.24 (br. s., 4H), 0.94-1.08 (m, 2H); ^{13}C NMR (126 MHz, DMSO) δ 169.7, 167.8, 162.3, 158.4, 158.2, 157.5, 157.2, 156.5, 156.4, 155.7, 136.5, 131.7, 130.0, 127.7, 119.4, 116.7, 114.4, 113.6, 73.8, 72.8, 60.7, 44.6, 42.6, 40.9, 40.8, 40.6, 37.3, 34.9, 29.9, 29.6, 22.1; LRMS (ESI) m/z 845.3 ($M+H^+$).

Determination of the off-rate of RG3039 from DcpS

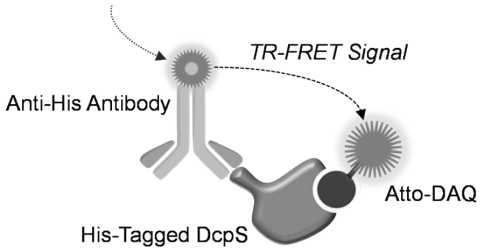

Figure S1. Diagram of the TR-FRET assay used to determine the offset kinetics of DcpS inhibitor RG3039

His-tagged DcpS (5 nM) in phosphate-buffered saline (75 mM KPO_4, 10 mM NaCl, pH 7.4) was preincubated with Tb-labelled anti-His antibody (6 nM) at room temperature for 30 minutes. RG3039 (10 nM) was added and after 30 minutes, Atto-DAQ (400 nM) was added. TR-FRET was monitored every 60 seconds (acceptor emission value at 520 nm/donor emission value at 490 nm) – ligand dissociation results in a signal increase due to Atto-DAQ binding.

Determination of DcpS occupancy using the SF chemical probe assay

DcpS occupancy in human PBMCs by RG3039 was measured as described previously[2] with slight modifications. Briefly, frozen human PBMCs (StemCell Technologies) were thawed, transferred to 10 mL RPMI medium with 10% FBS, centrifuged at 1200 rpm for 5 min, and then incubated in 10 mL RPMI medium with 10% FBS at 37 °C for 30 minutes. Cells were then washed with 10 mL PBS and resuspended in RPMI medium with 0.1% FBS. 1 mL was seeded in 1.5 mL eppendorf tubes with 25 million cells per tube. After incubation of the cells with various concentrations of RG3039 (0.3 nM to 1 μM) at room temperature for 1.5 hours, the sulfonyl fluoride probe SF-p1-yne was added (final concentration 1 μM) and incubated with cells at room temperature for 20 minutes with rotation. Cells were spun at 1500 x g for 1 minute, washed with 1 mL PBS, and then lysed in 50 μL PBS supplemented with protease inhibitors with brief sonication. Cell debris was removed by centrifugation for 1 minute at 16,000 x g. Click reaction with biotin azide was performed using Click-iT protein reaction kit (Life Technologies) following manufacturer's instructions. 500 μL of 6 M urea in PBS and 150 μL high-capacity streptavidin beads (Thermo Scientific) was added to the mixture and incubated at room temperature for 1 hour. Beads were washed thrice with 1 mL 3 M urea in PBS, and then eluted with 100 μL of 2x LDS sample buffer by boiling. The eluate was separated by SDS-PAGE, and analysed by immunoblot (α-DcpS antibody from Abcam). DcpS occupancy was calculated by comparing DcpS signals from RG3039 treated samples (measured using Kodak Imager) to the signal from the "no inhibitor" sample.

Determination of DcpS occupancy in cells using CETSA

HEK293 cells were incubated with DMSO or 1 μM D156844 at 37 °C for 30 min. The cells were then washed, resuspended with PBS, divided into 10 equal aliquots (2 million cells per aliquot), and then transferred to a PCR plate, and heated in Eppendorf MasterCycler (38.4 °C – 72.4 °C gradient) for 3 minutes. Cells were lysed in PBS with protease inhibitors by three freeze-thaw cycles in liquid nitrogen, and then spun at 16,000 g at 4 °C for 10 minutes. The supernatant was analysed by immunoblot using DcpS antibody after SDS-PAGE. CETSA curves were obtained by plotting percentage of soluble DcpS against temperature. ITDRF was carried out in the same fashion as described above, except that the cells were incubated with various concentrations of RG3039 or D156844 ranging from 0.3 nM to 1 μM), and the heating was performed at 65 °C.

References

[1] J. Singh, M. E. Gurney, **WO2008016973**.
[2] E. C. Hett, H. Xu, K. F. Geoghegan, A. Gopalsamy, R. E. Kyne Jr., C. A. Menard, A. Narayanan, M. D. Parikh, S. Liu, L. Roberts, R. P. Robinson, M. A. Tones, L. H. Jones, *ACS Chem. Biol.* **2015**, *10*, 1094-1098.