Characterization of an A-Site Selective Protein Disulfide Isomerase A1 Inhibitor

Kyle S. Cole[1], Julia M. D. Grandjean[2], Kenny Chen[3], Collin H. Witt[1], Johanna O'Day[1], Matthew D. Shoulders[3], R. Luke Wiseman[2], Eranthie Weerapana*[1]

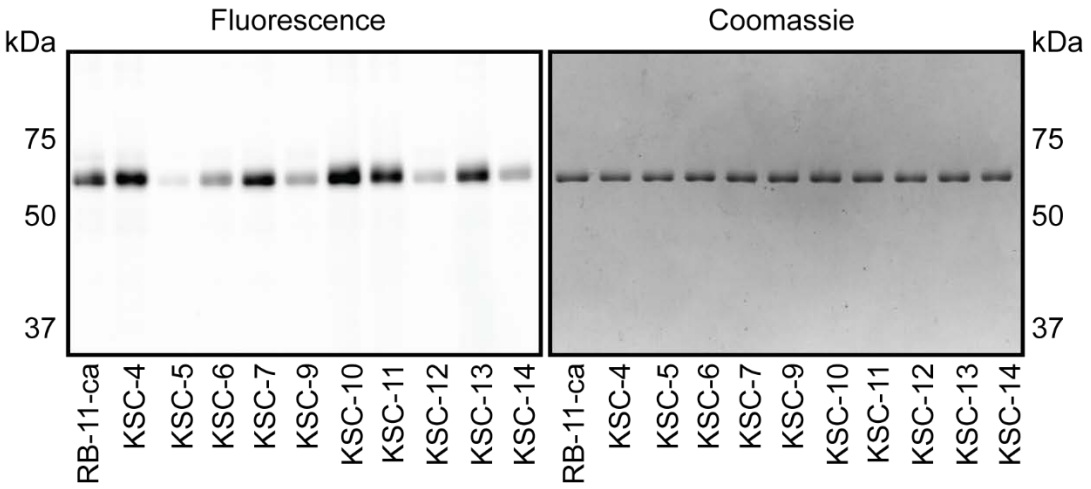
[1] Department of Chemistry, Boston College, Chestnut Hill, MA 02467, United States

[2] Department of Molecular Medicine, The Scripps Research Institute, La Jolla, CA 92037, United States

[3] Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139, United States

*Corresponding author: eranthie@bc.edu

Supporting Information



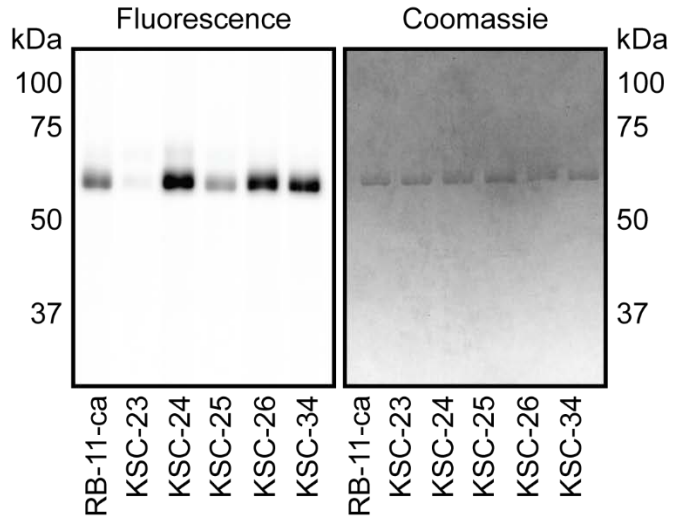


Figure S1. Characterization of PDIA1 second-generation library members. Library members (5 µM) were incubated with purified recombinant PDIA1 (50 µg/mL) in PBS, and protein labeling by each compound was evaluated after CuAAC-mediated incorporation of a fluorophore, SDS-PAGE, and in-gel fluorescence. Library members with equal or great potency than RB-11-ca progressed to the next step of the assay (Figure S2).

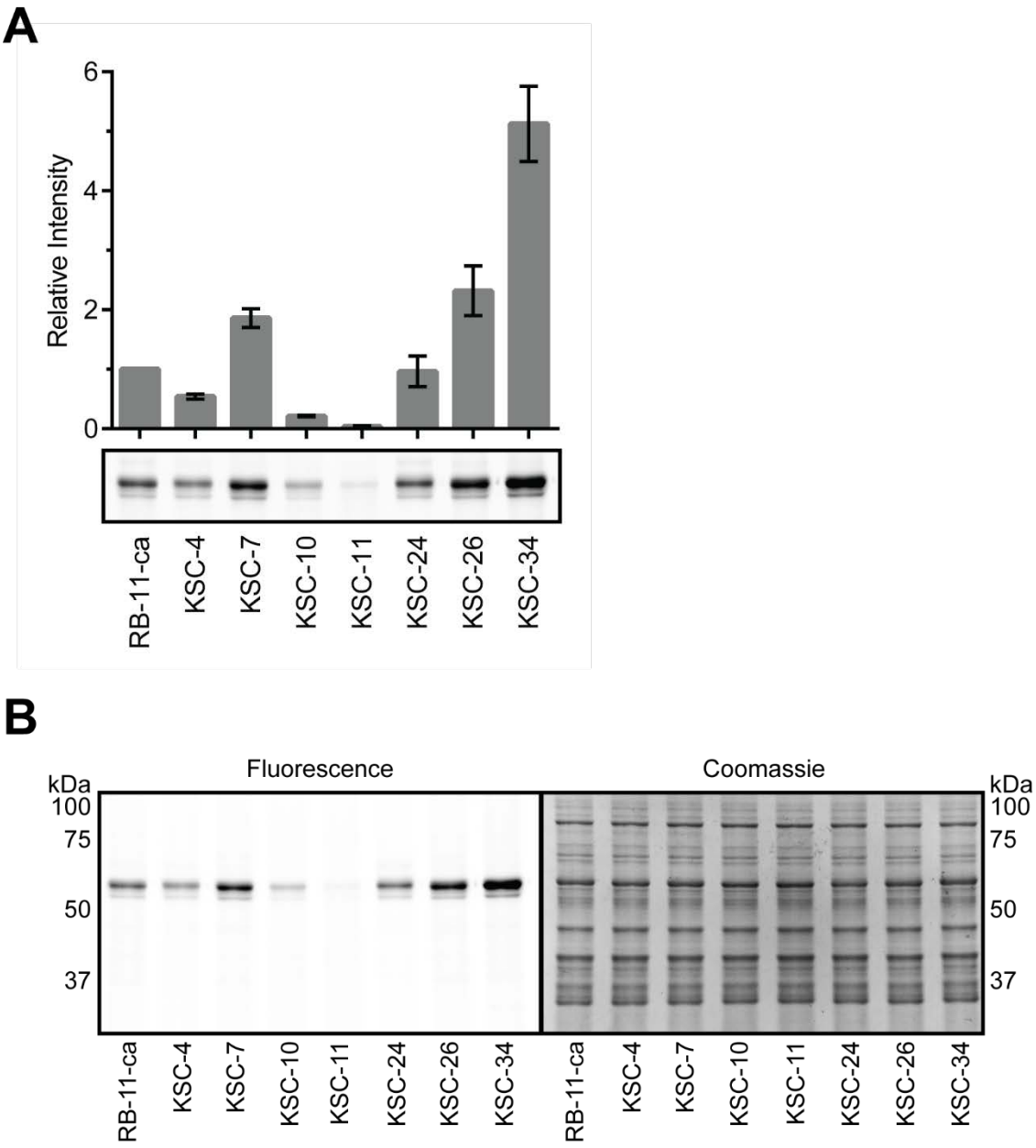


Figure S2. Characterization of PDIA1 second-generation library members. (A) Library members were assessed against purified, recombinant PDIA1 (50 µg/mL) in the background of MCF-7 cell lysates (1 mg/mL) to evaluate whether other cellular proteins interfere with the ability of each compound to covalently modify PDIA1. (B) Complete gels for part (A) illustrating proteome-wide selectivity of second-generation library members.

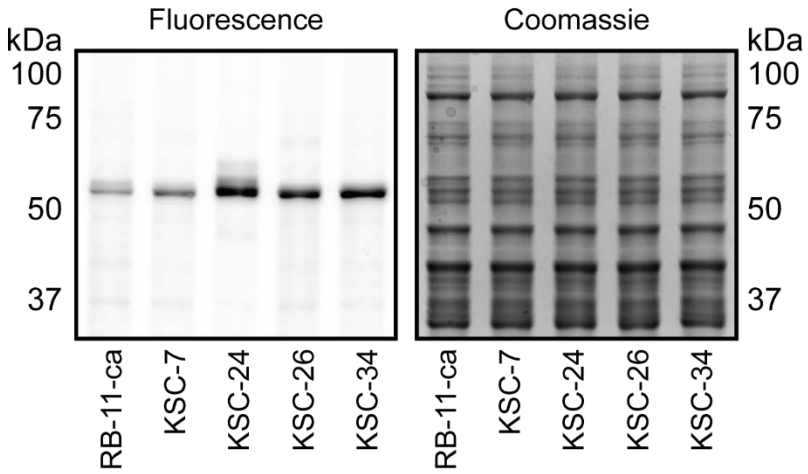


Figure S3. Characterization of PDIA1 second-generation library members. Library members were evaluated in MCF-7 lysates (2 mg/mL) for their ability to covalently modify endogenous PDIA1. The two most potent library members, KSC-24 and KSC-34, were advanced to the final screening step.

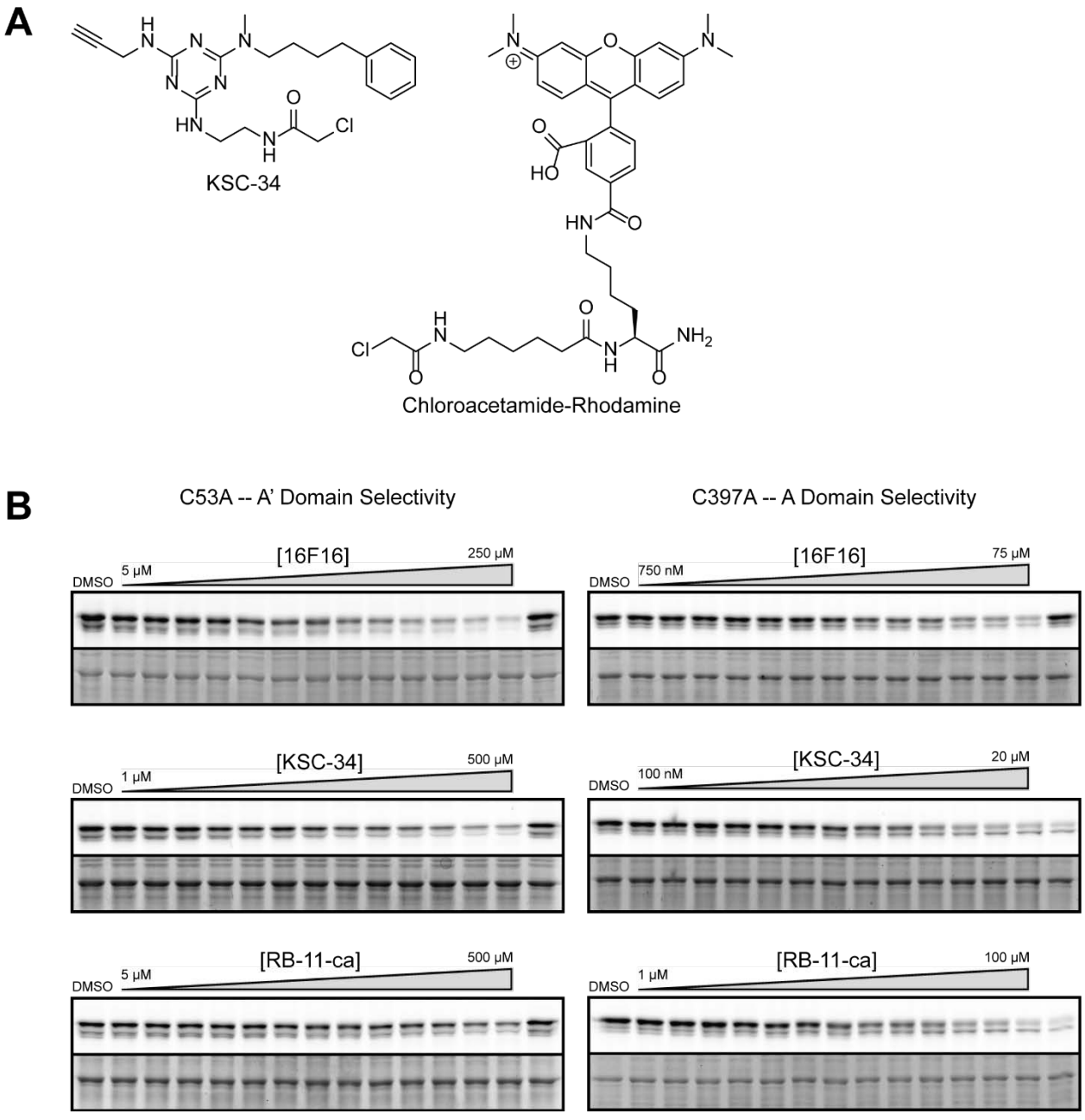


Figure S4. Assessing site-selectivity of PDIA1 inhibitors. (A) Structures of KSC-34 and Chloroacetamide-Rhodamine (CA-Rh). (B) PDIA1 C53A and C397A (50 µg/mL) in the background of MCF-7 lysates (1 mg/mL) were treated with increasing concentrations of KSC-34, RB-11-ca and 16F16 to quantify the selectivity for the **a** and **a'** domains. Following incubation, samples were then treated with CA-Rh to append a fluorophore to the residual, unmodified PDIA1 active sites. Loss of fluorescence indicates inhibitor binding. ImageJ (NIH) was used to quantify the loss in fluorescence and calculate affinities for each active site.

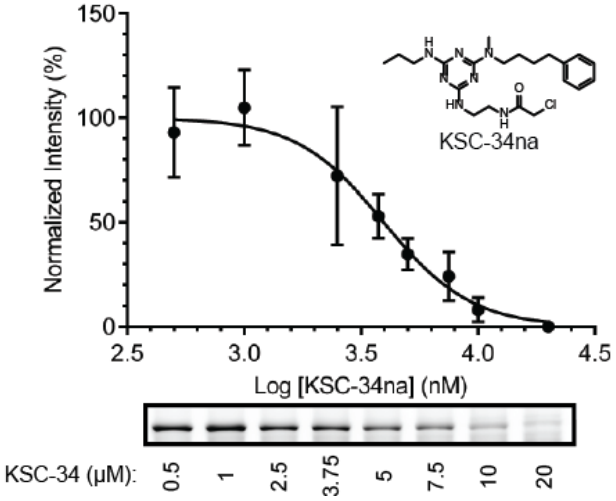


Figure S5. Identification of KSC-34 cellular occupancy of PDIA1. MCF-7 cells were treated with varying concentrations of KSC-34na. Following lysis, protein concentrations were normalized and subjected to treatment with KSC-34 (5 μM) to modify any residual PDIA1 **a** domain active site, C53. ImageJ was used to quantify the amount of PDIA1 that was not occupied by KSC-34na at each concentration. An EC_{50} of ~4 μM was calculated for KSC-34na labeling of PDIA1 in MCF-7 whole cells.

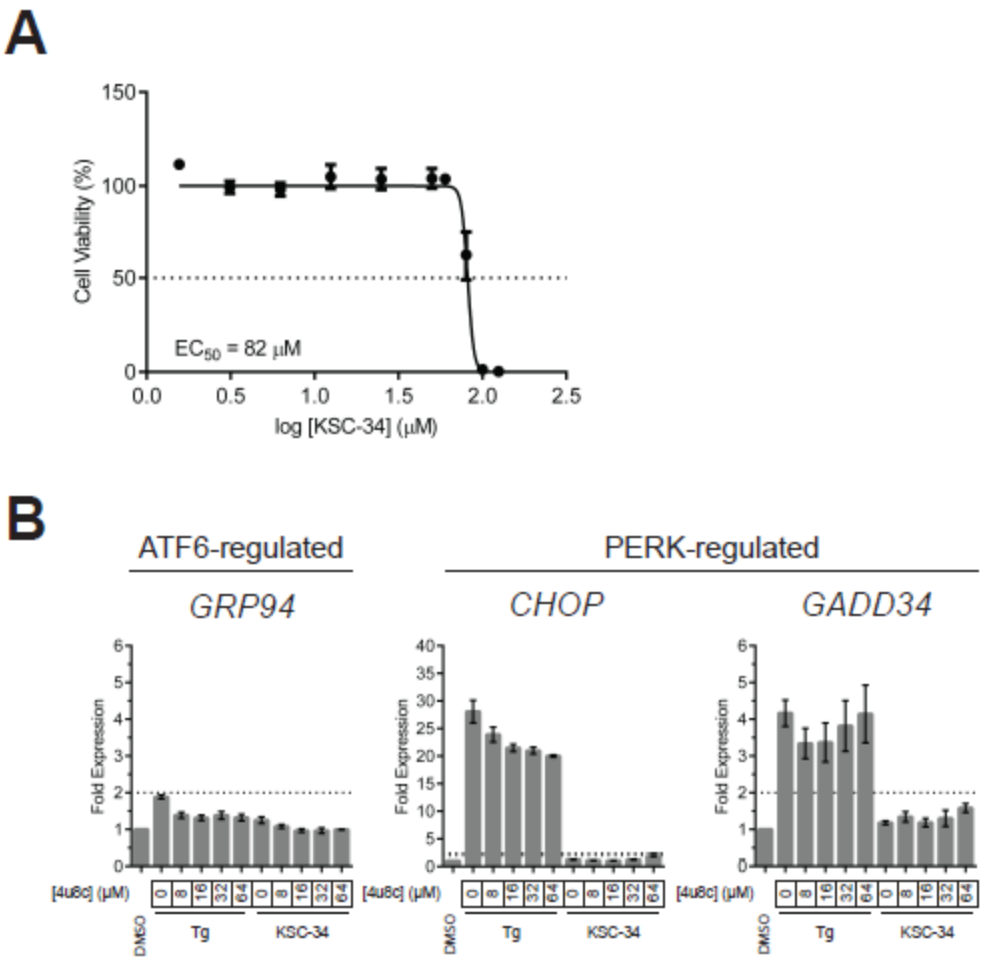


Figure S6. Effects of KSC-34 on cell viability and the unfolded protein response. (A) MTT assay data in MCF-7 cells treated with varying concentrations of KSC-34. The calculated EC50 of KSC-34 was determined to be 82 µM. Error bars represent SD for n=3 experiments. (B) qPCR analysis of UPR target genes following co-treatment of MCF-7 cells with KSC-34 (20 µM) and IRE1α inhibitor, 4u8c. qPCR data are reported relative to the corresponding DMSO-treated cells +/- SEM from n=3 biological replicates.

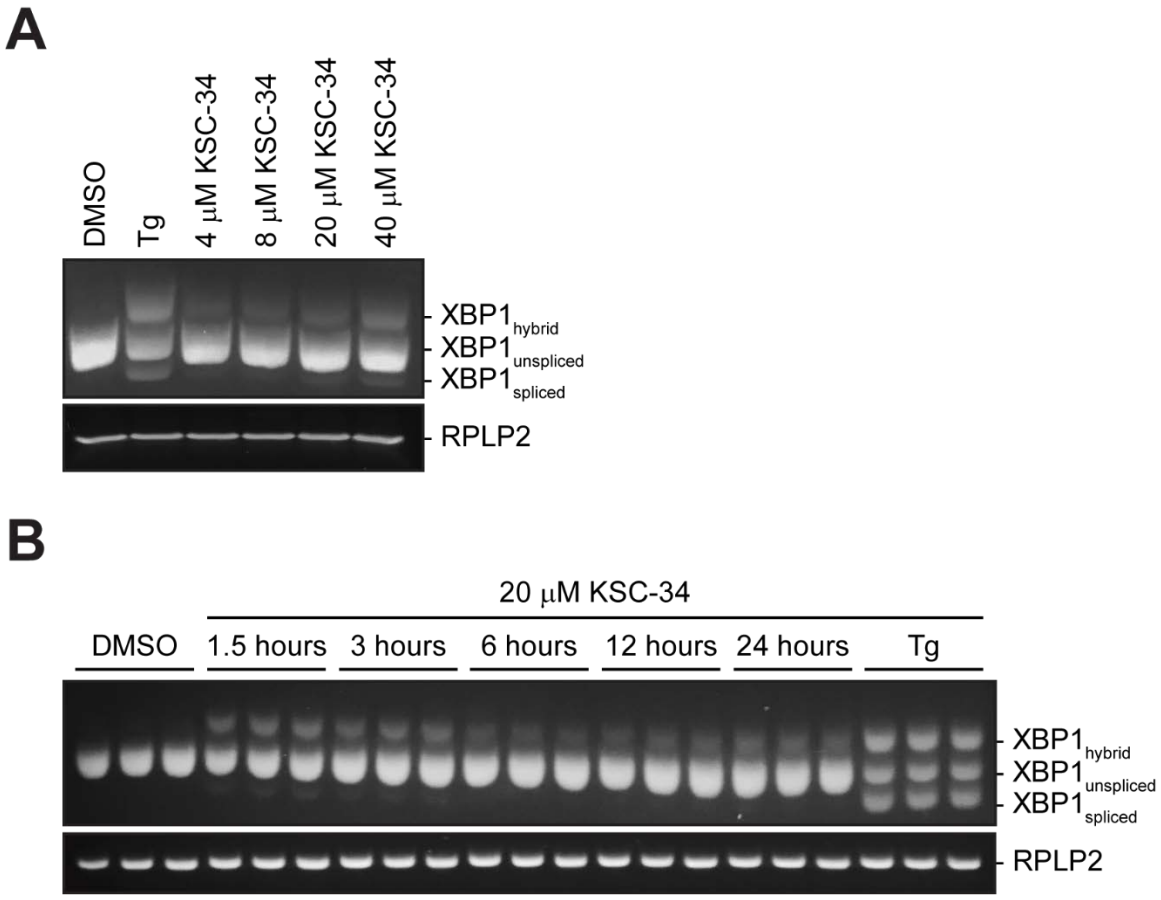


Figure S7. XBP1 splicing assay. Cells were lysed in tissue culture plates, and total RNA was extracted using the Omega E.Z.N.A. Total RNA Kit I according to the manufacturer's instructions. cDNA synthesized from total RNA was amplified using the Q5 High-Fidelity DNA Polymerase (NEB) with the appropriate *XBP1* primers (Table 1). Five microliters of the resultant reaction were separated on 2.5% agarose gels stained with GelGreen (Biotium). (A) XBP1 splicing assay for MCF-7 cells treated with varying concentrations of KSC-34 for 3 hours. (B) XBP1 splicing assay for time-course experiment, where MCF-7 cells were treated with 20 μM KSC-34. Gel shows three biological replicates for each condition.

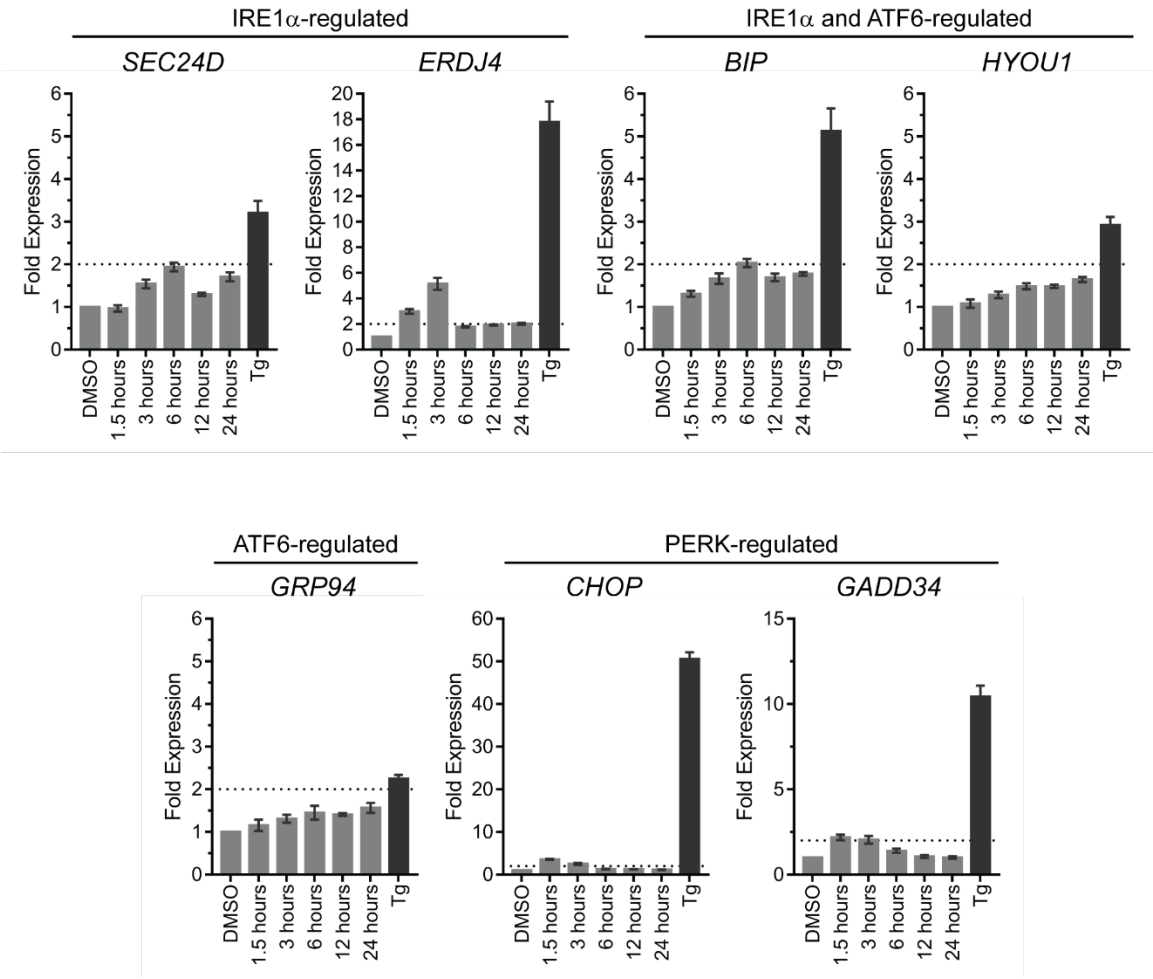


Figure S8. Activation of UPR target genes in MCF-7 cells was assessed by measuring the relative mRNA expression levels of target genes by qRT-PCR after treating with KSC-34 (20 μM) for increasing time. Transcripts were normalized to the housekeeping gene RPLP2, and all reactions were performed in technical quadruplicate. Data are reported as the mean fold change (relative to DMSO) ± SEM from three biological replicates.

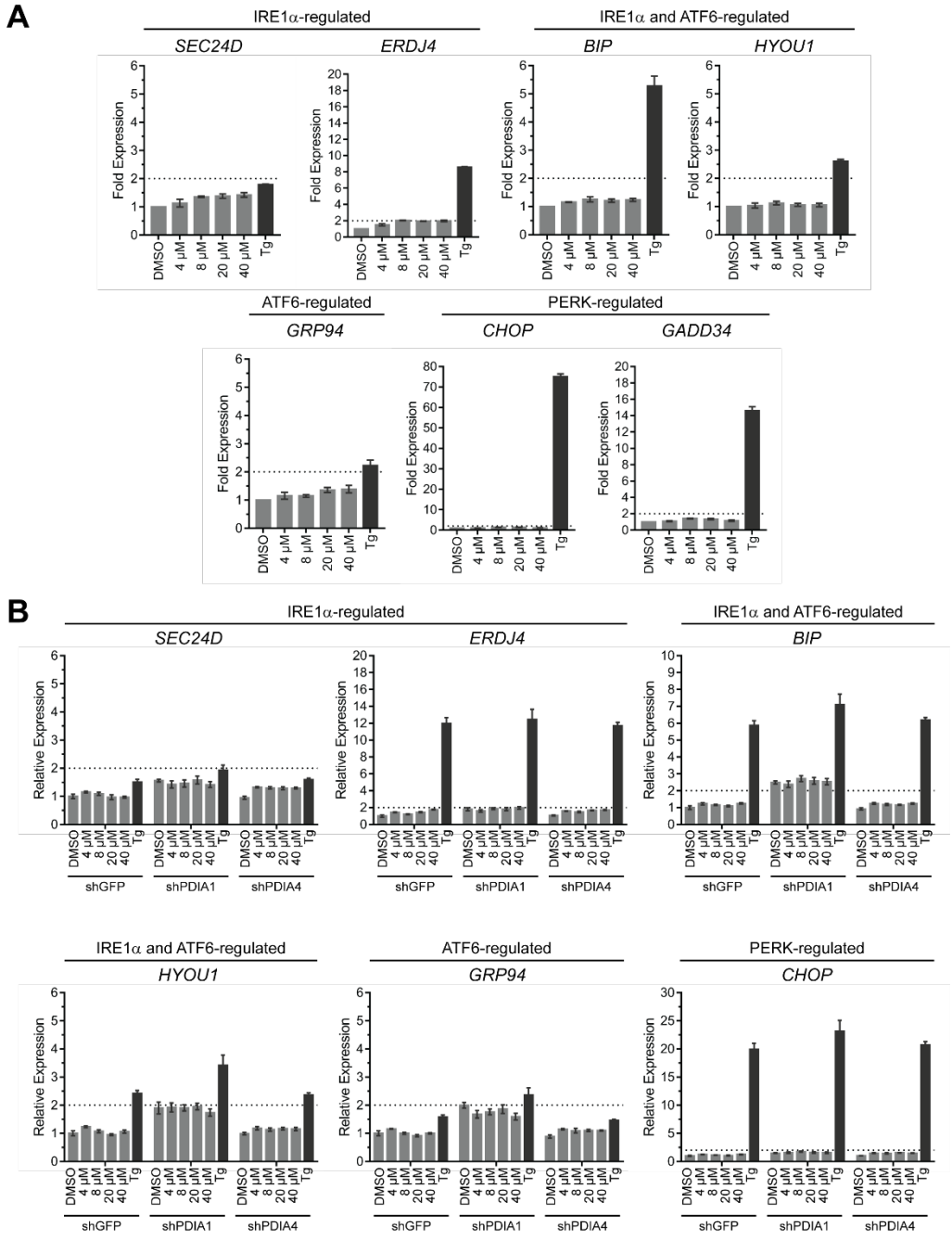


Figure S9. Activation of UPR target genes in A549 cells (A) or SKOV-3 cells stably expressing shPDIA1, shPDIA4 or shGFP (B). The relative mRNA expression levels of target genes were assessed by qRT-PCR after treating with increasing concentrations of KSC-34 or 5 μM of thapsigargin. Transcripts were normalized to the housekeeping gene RPLP2. (A) For A549 cells, all reactions were performed in technical quadruplicate, and data are reported as the mean fold change (relative to DMSO) ± SEM from three biological replicates. (B) For SKOV-3 cells, data are reported as mean fold change (relative to shGFP DMSO) ± SEM from reactions performed in triplicate.

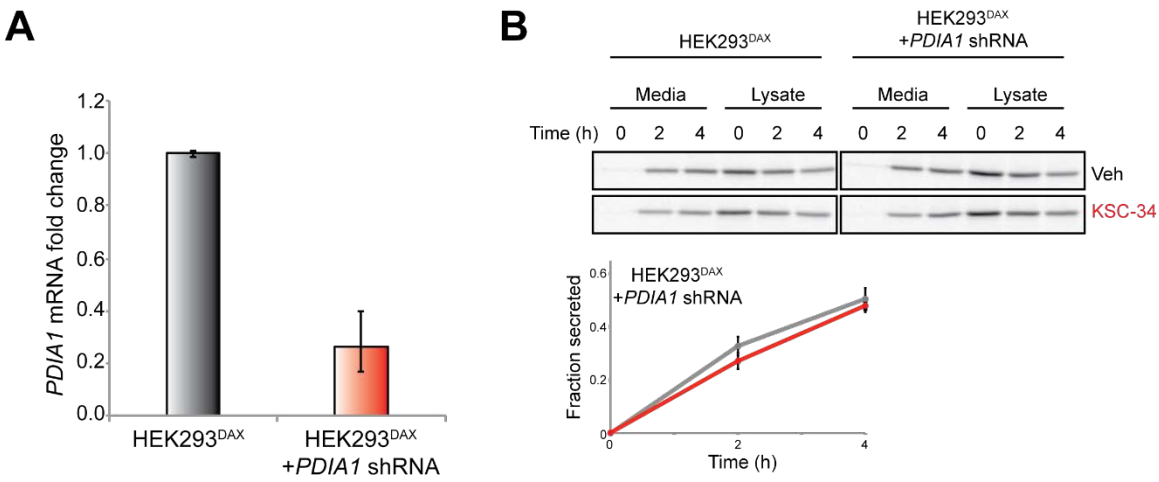


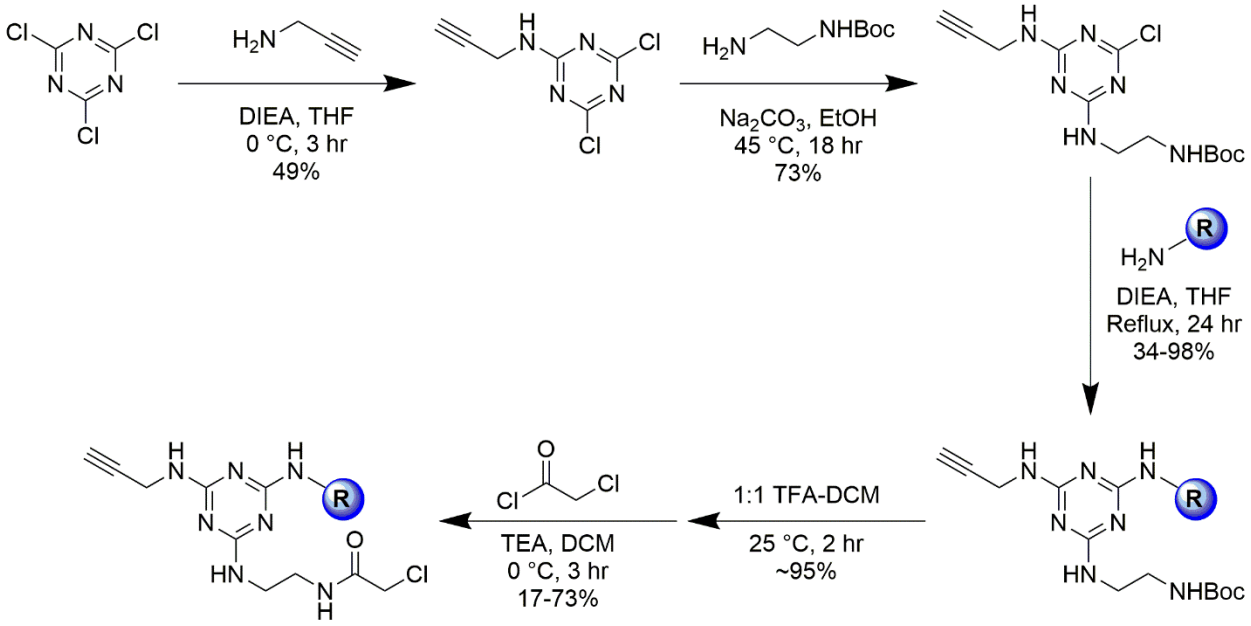
Figure S10. (A) Graph showing fold change mRNA levels of *PDIA1* in HEK293^{DAX} cells and HEK293^{DAX} cells shRNA-depleted of *PDIA1* measured by qPCR. Error bars show 95% confidence interval. (B) Representative autoradiogram and quantification of the fraction [^{35}S]-labeled ^{FT}ALLC secreted from HEK293^{DAX} cells using the experimental paradigm shown in **Figure 6C**. Experiments were performed in the absence or presence of KSC-34 (40 μM) added 1 h prior to labeling and then again throughout the experiment. Fraction secreted was calculated as described in Materials and Methods (*). Error bars show SEM for n=4.

Materials and Methods

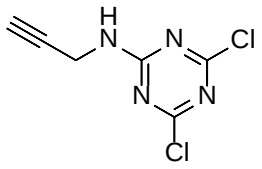
1. General Information

All materials were obtained from Sigma-Aldrich, Fisher Scientific, Combi-Blocks, or Oakwood Chemicals, unless otherwise noted. Phosphate buffered saline (PBS) buffer, RPMI 1640 media, Trypsin-EDTA and Anti-Anti were purchased from Fisher Scientific (Pittsburgh, PA). Fetal bovine serum was purchased from Atlanta Biologicals (Flowery Branch, GA). All protein concentrations were determined using the DC Protein Assay kit from Bio-Rad (Hercules, CA). Analytical thin layer chromatography (TLC) was performed on EMD Millipore F_{254} glass-backed TLC plates (250 µm, Billerica, MA). All compounds were visualized on TLC under UV light and by potassium permanganate staining. Column chromatography was carried out using forced flow of solvent on Sorbent Technologies (Norcross, GA) standard grade silica gel, 40-63 µm particle size, 60 Å pore size. Proton and carbon NMR spectra were carried out on Varian (Palo Alto, CA) 500 MHz and 600 MHz NMR spectrometers. Chemical shifts (δ) are reported in parts per million (ppm) with chemical shifts reported to internal standards: $CDCl_3$ (7.26 ppm for ^1H, 77.23 ppm for ^{13}C), $(CD_3)_2CO$ (2.05 ppm for ^1H, 29.92 ppm for ^{13}C), CD_3OD (3.31 ppm for ^1H, 49.15 ppm for ^{13}C). Coupling constants (*J*) are reported in Hertz (Hz) and multiplicities are abbreviated as singlet (s), doublet (d), triplet (t), multiplet (m), doublet of doublets (dd), and doublet of triplets (dt). High resolution Mass Spectra (HRMS) were obtained at the Mass Spectrometry Facility at Boston College (Chestnut Hill, MA).

2. Synthetic Methods and Characterization

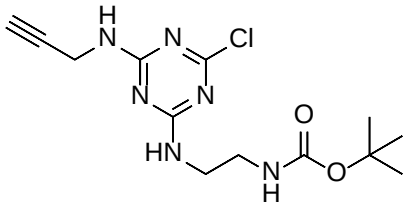


Scheme S1. General synthetic route for triazine-based covalent PDIA1 inhibitors.



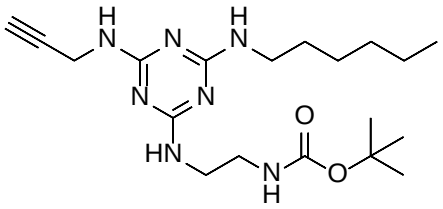
BBI

4,6-dichloro-N-(prop-2-yn-1-yl)-1,3,5-triazin-2-amine (BBI): To an ice cold solution of cyanuric chloride (1.0 eq, 151.1 mg, 0.8194 mmol) and diisopropylethylamine (1.1 eq, 116.5 mg, 0.9013 mmol) in tetrahydrofuran (THF) (0.055 M, 15 mL), propargylamine (1.2 eq, 54.2 mg, 0.9832 mmol) was added dropwise. The reaction mixture was allowed to stir at 0 °C for 3 hours. The solvent was removed *in vacuo* and the crude product was purified via flash chromatography to give the desired product **BBI** as a white solid (82.2 mg, 49%). ^{1}H NMR (600 MHz, Acetone-d_6) δ 8.30 (s, 1H), 4.43 – 4.15 (m, 2H), 2.79 (t, J = 2.6 Hz, 1H). ^{13}C NMR (151 MHz, Acetone-d_6) δ 171.32, 170.63, 166.91, 79.56, 73.13, 31.35. HRMS for BBI: m/z calcd. 202.9813; obsd. 201.9901.



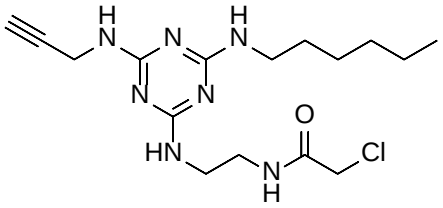
BBII

***tert*-butyl(2-((4-chloro-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)carbamate (BBII):** To a solution of **BBI** (1.0 eq, 79.4 mg, 0.3911 mmol) and sodium carbonate (2.2 eq, 91.2 mg, 0.8604 mmol) in ethanol (0.08 M, 5 mL), N-Boc-ethylenediamine (1.1eq, 68.9 mg, 0.4302 mmol) was added dropwise at room temperature. The solution was then allowed to warm to 45 °C while stirring and continued to stir for 18 hours. After the reaction was complete, 15 mL of water was added and the reaction was extracted with ethyl acetate (3 x 15 mL). The organic layer was washed with brine and dried with $MgSO_4$, then the solvent was removed *in vacuo*. The crude product was purified via flash chromatography to afford the desired to product, **BBII** as a white solid (93.2 mg, 73%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.12 (dt, J = 44.7, 5.8 Hz, 1H), 8.01 – 7.54 (m, 1H), 6.82 (dt, J = 11.4, 5.9 Hz, 1H), 4.17 – 3.92 (m, 2H), 3.31 – 3.19 (m, 2H), 3.18 – 3.00 (m, 2H), 2.50 (p, J = 1.8 Hz, 1H), 1.36 (d, J = 1.9 Hz, 9H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 168.38, 168.12, 167.78, 165.36, 164.79, 155.57, 80.87, 77.61, 72.91, 29.63, 28.21. HRMS for BBII: m/z calcd. 327.1258; obsd. 327.1345.



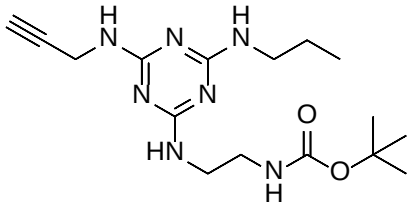
KSC-4-int

***tert*-butyl(2-((4-(hexylamino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)carbamate (KSC-4-int):** To a solution of **BBII** (1.0 eq, 92.8 mg, 0.2840 mmol) and diisopropylethylamine (2.5 eq, 91.8 mg, 0.7100 mmol) in THF (0.04 M, 8 mL), hexylamine (2.5 eq, 71.8 mg, 0.7100 mmol) was added dropwise at room temperature. The solution was then allowed to heat to reflux while stirring and continued to reflux for 24 hours. After the reaction was complete, the solvent was removed *in vacuo*. The crude product was purified via flash chromatography to afford the desired to product, **KSC-4-int** as an oil (107.2 mg, 96%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 6.01 – 4.66 (m, 4H), 4.16 (s, 2H), 3.60 – 3.16 (m, 6H), 2.27 – 2.12 (m, 1H), 1.52 (q, *J* = 7.4 Hz, 2H), 1.42 (d, *J* = 2.0 Hz, 9H), 1.37 – 1.25 (m, 6H), 0.87 (dt, *J* = 6.9, 3.3 Hz, 3H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 166.54, 165.75, 156.33, 81.00, 79.27, 71.01, 41.53, 40.84, 40.70, 31.67, 30.44, 29.82, 28.52, 26.73, 22.71, 14.22, 14.15. HRMS for KSC-4-int: m/z calcd. 392.2696; obsd. 392.2774.



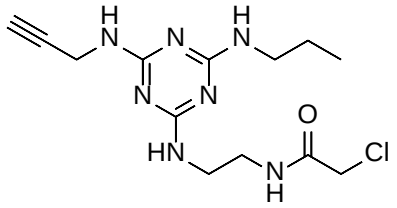
KSC-4

2-chloro-N-(2-((4-(hexylamino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)acetamide (KSC-4): KSC-4-int was deprotected using a 50% solution of TFA/DCM (4 mL) at 25 °C for 2 hours. The solvent was removed *in vacuo* and the crude product remained on the vacuum overnight to remove any excess TFA. Then, to an ice cold solution of deprotected amine (1.0 eq, 39.9 mg, 0.1369 mmol) and triethylamine (1.5 eq, 20.8 mg, 0.2054 mmol) in DCM (0.03 M, 5 mL), chloroacetyl chloride (1.2 eq, 18.6 mg, 0.1643 mmol) in DCM (2 mL) was added dropwise over 30 min. The reaction was stirred at 0 °C for 3 hours. After the reaction was complete, the solvent was removed *in vacuo* and the crude product was purified by flash chromatography to give the desired product **KSC-4** (26.3 mg, 52%). ^{1}H NMR (600 MHz, Chloroform-*d*) δ 4.21 (s, 2H), 4.03 (s, 2H), 3.74 – 3.43 (m, 2H), 3.39 (s, 2H), 2.23 (s, 1H), 1.56 (s, 2H), 1.31 (dd, *J* = 16.3, 9.6 Hz, 6H), 1.25 (d, *J* = 2.4 Hz, 2H), 0.88 (t, *J* = 6.7 Hz, 3H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 166.86, 163.97, 117.49, 115.56, 79.67, 71.63, 42.71, 41.06, 39.81, 31.60, 30.43, 29.83, 29.55, 26.64, 22.69, 14.15. HRMS for KSC-4: m/z calcd. 368.1887; obsd. 368.1966.



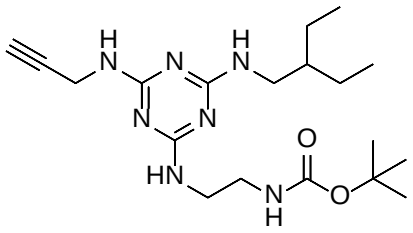
KSC-5-int

***tert*-butyl(2-((4-(prop-2-yn-1-ylamino)-6-(propylamino)-1,3,5-triazin-2-yl)amino)ethyl)carbamate (KSC-5-int):** To a solution of **BBII** (1.0 eq, 44.0 mg, 0.1346 mmol) and diisopropylethylamine (2.4 eq, 41.8 mg, 0.3231 mmol) in THF (0.03 M, 5 mL), propylamine (2.4 eq, 19.1 mg, 0.3231 mmol) was added dropwise at room temperature. The solution was then allowed to heat to reflux while stirring and continued to reflux for 24 hours. After the reaction was complete, the solvent was removed *in vacuo*. The crude product was purified via flash chromatography to afford the desired to product, **KSC-5-int** as an oil (37.4 mg, 80%). ^{1}H NMR (600 MHz, Chloroform-*d*) δ 4.24 – 4.06 (m, 2H), 3.47 (s, 2H), 3.29 (s, 4H), 2.20 (t, *J* = 2.5 Hz, 1H), 1.56 (q, *J* = 7.5 Hz, 2H), 1.41 (s, 9H), 0.93 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 166.70, 166.15, 165.69, 156.32, 81.03, 79.27, 71.00, 42.60, 41.67, 40.68, 30.47, 28.53, 23.11, 11.58. HRMS for KSC-5-int: m/z calcd. 350.2226; obsd. 350.2324.



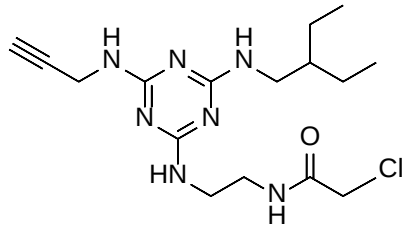
KSC-5

2-chloro-N-(2-((4-(prop-2-yn-1-ylamino)-6-(propylamino)-1,3,5-triazin-2-yl)amino)ethyl)acetamide (KSC-5): KSC-5-int was deprotected using a 50% solution of TFA/DCM (4 mL) at 25 °C for 2 hours. The solvent was removed *in vacuo* and the crude product remained on the vacuum overnight to remove any excess TFA. Then, to an ice cold solution of deprotected amine (1.0 eq, 31.3 mg, 0.1256 mmol) and triethylamine (1.5 eq, 19.0 mg, 0.1884 mmol) in DCM (0.025 M, 5 mL), chloroacetyl chloride (1.2 eq, 17.0 mg, 0.1507 mmol) in DCM (2 mL) was added dropwise over 30 min. The reaction was stirred at 0 °C for 3 hours. After the reaction was complete, the solvent was removed *in vacuo* and the crude product was purified by flash chromatography to give the desired product **KSC-5** (23.5 mg, 58%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 4.30 – 4.12 (m, 2H), 4.04 (s, 2H), 3.73 – 3.22 (m, 6H), 2.30 – 2.21 (m, 1H), 1.35 – 1.15 (m, 2H), 0.95 (d, *J* = 7.8 Hz, 3H). ^{13}C NMR (151 MHz, Acetone-d_6) δ 167.50, 163.27, 152.84, 76.67, 74.19, 72.96, 51.02, 45.41, 43.36, 41.60, 40.25, 39.27, 37.53, 24.38, 23.33, 14.36, 11.23. HRMS for KSC-5: m/z calcd. 326.1418; obsd. 326.1487.



KSC-6-int

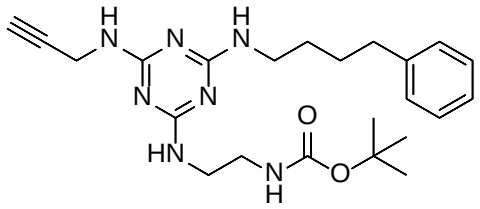
***tert*-butyl(2-((4-((2-ethylbutyl)amino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)carbamate (KSC-6-int):** To a solution of **BBII** (1.0 eq, 47.1 mg, 0.1441 mmol) and diisopropylethylamine (2.4 eq, 44.7 mg, 0.3459 mmol) in THF (0.03 M, 5 mL), 2-ethylbutylamine (2.4 eq, 35.0 mg, 0.3459 mmol) was added dropwise at room temperature. The solution was then allowed to heat to reflux while stirring and continued to reflux for 24 hours. After the reaction was complete, the solvent was removed *in vacuo*. The crude product was purified via flash chromatography to afford the desired to product, **KSC-6-int** as an oil (46.4 mg, 82%). ^{1}H NMR (600 MHz, Chloroform-*d*) δ 4.17 (s, 2H), 3.48 (s, 2H), 3.29 (s, 4H), 2.20 (t, *J* = 2.6 Hz, 1H), 1.42 (s, 9H), 1.33 (p, *J* = 7.2 Hz, 4H), 1.25 (d, *J* = 2.7 Hz, 1H), 0.89 (t, *J* = 7.4 Hz, 6H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 166.38, 165.59, 156.32, 81.00, 79.29, 71.01, 43.15, 41.71, 41.27, 40.70, 30.52, 29.83, 23.92, 14.25, 11.15. HRMS for KSC-6-int: m/z calcd. 392.2696; obsd. 392.2778.



KSC-6

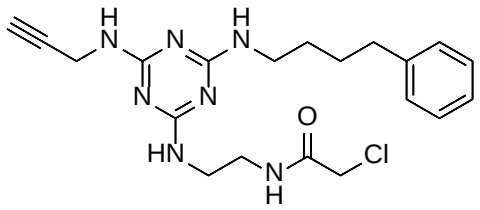
2-chloro-N-(2-((4-((2-ethylbutyl)amino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)acetamide (KSC-6): KSC-6-int was deprotected using a 50% solution of TFA/DCM (4 mL) at 25 °C for 2 hours. The solvent was removed *in vacuo* and the crude product remained on the vacuum overnight to remove any excess TFA. Then, to an ice cold solution of deprotected amine (1.0 eq, 34.5 mg, 0.1185 mmol) and triethylamine (1.5 eq, 18.0 mg, 0.1778 mmol) in DCM (0.025 M, 5 mL), chloroacetyl chloride (1.2 eq, 16.1 mg, 0.1422 mmol) in DCM (2 mL) was added dropwise over 30 min. The reaction was stirred at 0 °C for 3 hours. After the reaction was complete, the solvent was removed *in vacuo* and the crude product was purified by flash chromatography to give the desired product **KSC-6** (31.0 mg, 71%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 4.28 – 4.10 (m, 2H), 4.04 (s, 2H), 3.71 – 3.22 (m, 4H), 2.32 – 2.20 (m, 1H), 1.42 – 1.30 (m, 10H), 1.25 (d, *J* = 2.4 Hz, 3H). ^{13}C NMR (151 MHz, Acetone-d_6) δ 167.50, 163.27,

152.84, 76.67, 74.19, 72.96, 51.02, 45.41, 43.36, 41.60, 40.25, 39.27, 37.53, 24.38, 23.33, 14.36, 11.23. HRMS for KSC-6: m/z calcd. 368.1887; obsd. 368.1976.



KSC-7-int

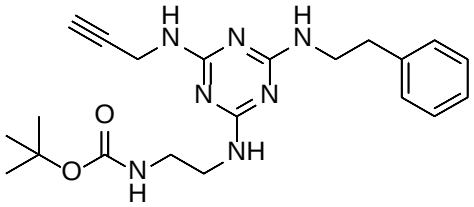
***tert*-butyl(2-((4-((4-phenylbutyl)amino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)carbamate (KSC-7-int):** To a solution of **BBII** (1.0 eq, 53.9 mg, 0.1649 mmol) and diisopropylethylamine (2.4 eq, 51.1 mg, 0.3956 mmol) in THF (0.03 M, 5 mL), 4-phenylbutylamine (2.4 eq, 59.0 mg, 0.3956 mmol) was added dropwise at room temperature. The solution was then allowed to heat to reflux while stirring and continued to reflux for 24 hours. After the reaction was complete, the solvent was removed *in vacuo*. The crude product was purified via flash chromatography to afford the desired to product, **KSC-7-int** as an oil (70.7 mg, 98%). ^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.30 – 7.23 (m, 2H), 7.17 (t, *J* = 8.1 Hz, 3H), 4.31 – 3.99 (m, 2H), 3.53 – 3.13 (m, 6H), 2.63 (t, *J* = 7.6 Hz, 2H), 2.19 (s, 1H), 1.67 (q, *J* = 7.8 Hz, 2H), 1.58 (t, *J* = 7.8 Hz, 2H), 1.42 (s, 9H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 166.15, 156.31, 142.34, 128.52, 125.88, 81.01, 79.29, 71.02, 41.61, 40.71, 40.61, 35.69, 30.49, 29.83, 29.54, 28.81, 28.54, 14.33, 1.15. HRMS for KSC-7-int: m/z calcd. 440.2696; obsd. 440.2739.



KSC-7

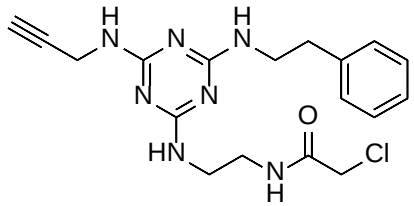
2-chloro-N-(2-((4-((4-phenylbutyl)amino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)acetamide (KSC-7): KSC-7-int was deprotected using a 50% solution of TFA/DCM (4 mL) at 25 °C for 2 hours. The solvent was removed *in vacuo* and the crude product remained on the vacuum overnight to remove any excess TFA. Then, to an ice cold solution of deprotected amine (1.0 eq, 54.6 mg, 0.1608 mmol) and triethylamine (1.5 eq, 24.4 mg, 0.2412 mmol) in DCM (0.03 M, 5 mL), chloroacetyl chloride (1.2 eq, 21.8 mg, 0.1930 mmol) in DCM (2 mL) was added dropwise over 30 min. The reaction was stirred at 0 °C for 3 hours. After the reaction was complete, the solvent was removed *in vacuo* and the crude product was purified by flash chromatography to give the desired product **KSC-7** (47.9 mg, 72%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.31 – 7.24 (m, 2H), 7.23 – 7.10 (m, 3H), 4.29 – 4.09 (m, 2H), 4.06 (s, 2H), 3.71

– 3.31 (m, 4H), 2.71 – 2.56 (m, 1H), 1.65 (d, *J* = 16.0 Hz, 4H), 1.41 – 1.17 (m, 4H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 167.00, 141.93, 128.52, 126.04, 78.42, 72.40, 53.56, 42.72, 41.52, 40.83, 40.01, 39.34, 35.54, 31.33, 30.30, 29.84, 28.63, 22.83. HRMS for KSC-7: m/z calcd. 416.1887; obsd. 416.1963.



KSC-9-int

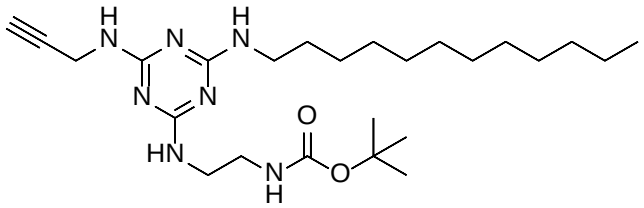
***tert*-butyl(2-((4-(phenethylamino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)carbamate (KSC-9-int):** To a solution of **BBII** (1.0 eq, 92.8 mg, 0.2840 mmol) and diisopropylethylamine (4.0 eq, 146.8 mg, 1.1359 mmol) in THF (0.05 M, 5 mL), 2-phenethylamine hydrochloride (4.0 eq, 137.7 mg, 1.1359 mmol) was added dropwise at room temperature. The solution was then allowed to heat to reflux while stirring and continued to reflux for 24 hours. After the reaction was complete, the solvent was removed *in vacuo*. The crude product was purified via flash chromatography to afford the desired to product, **KSC-9-int** as an oil (57.1 mg, 49%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.29 (t, *J* = 7.6 Hz, 2H), 7.20 (d, *J* = 7.2 Hz, 3H), 5.82 – 4.91 (m, 4H), 4.15 (d, *J* = 17.4 Hz, 2H), 3.74 – 3.38 (m, 4H), 3.29 (s, 2H), 2.86 (t, *J* = 7.4 Hz, 2H), 2.19 (s, 1H), 1.41 (s, 9H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 166.87, 166.31, 165.96, 156.58, 139.64, 129.17, 126.70, 81.29, 79.56, 71.27, 42.43, 40.95, 36.40, 30.71, 30.06, 28.78, 23.06. HRMS for KSC-9-int: m/z calcd. 412.2383; obsd. 412.2461.



KSC-9

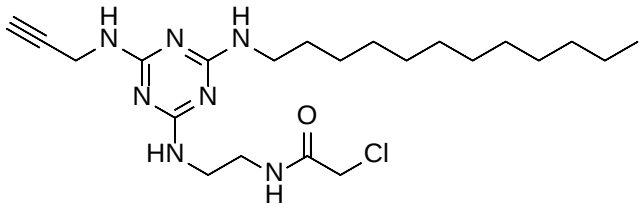
2-chloro-N-(2-((4-(phenethylamino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)acetamide (KSC-9): KSC-9-int was deprotected using a 50% solution of TFA/DCM (4 mL) at 25 °C for 2 hours. The solvent was removed *in vacuo* and the crude product remained on the vacuum overnight to remove any excess TFA. Then, to an ice cold solution of deprotected amine (1.0 eq, 125.5 mg, 0.4030 mmol) and triethylamine (1.3 eq, 53.0 mg, 0.5239 mmol) in DCM (0.08 M, 5 mL), chloroacetyl chloride (1.3 eq, 59.2 mg, 0.5239 mmol) in DCM (2 mL) was added dropwise over 30 min. The reaction was stirred at 0 °C for 3 hours. After the reaction was complete, the solvent was removed *in vacuo* and the crude product was purified by

flash chromatography to give the desired product **KSC-9** (26.9 mg, 17%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.46 – 7.28 (m, 3H), 7.25 – 7.18 (m, 2H), 4.23 (m, 2H), 4.16 – 3.98 (m, 2H), 3.79 – 3.45 (m, 6H), 2.90 (dt, *J* = 17.7, 8.8 Hz, 2H), 2.36 – 2.22 (m, 1H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 174.40, 156.18, 137.95, 128.60, 126.62, 101.36, 72.19, 49.42, 42.32, 39.84, 39.20, 35.38, 31.04, 30.06, 29.60, 14.01. HRMS for KSC-9: m/z calcd. 388.1574; obsd. 388.1653.



KSC-10-int

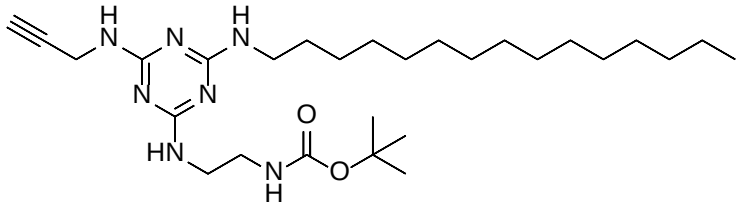
***tert*-butyl(2-((4-(dodecylamino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)carbamate (KSC-10-int):** To a solution of **BBII** (1.0 eq, 51.6 mg, 0.1580 mmol) and diisopropylethylamine (2.5 eq, 51.1 mg, 0.3950 mmol) in THF (0.03 M, 5 mL), dodecylamine (2.5 eq, 73.2 mg, 0.3950 mmol) was added dropwise at room temperature. The solution was then allowed to heat to reflux while stirring and continued to reflux for 24 hours. After the reaction was complete, the solvent was removed *in vacuo*. The crude product was purified via flash chromatography to afford the desired to product, **KSC-10-int** as an oil (53.5 mg, 71%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.78 – 4.84 (m, 4H), 4.15 (s, 2H), 3.57 – 3.18 (m, 6H), 2.20 (t, *J* = 2.5 Hz, 1H), 1.59 – 1.46 (m, 2H), 1.41 (s, 9H), 1.37 – 1.17 (m, 18H), 0.87 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 165.93, 156.55, 134.07, 129.61, 93.58, 81.10, 79.53, 71.29, 41.78, 41.09, 32.26, 30.74, 30.08, 30.01, 29.97, 29.94, 29.73, 29.69, 28.75, 27.29, 23.03, 14.46, 1.36. HRMS for KSC-10-int: m/z calcd. 476.3635; obsd. 476.3692.



KSC-10

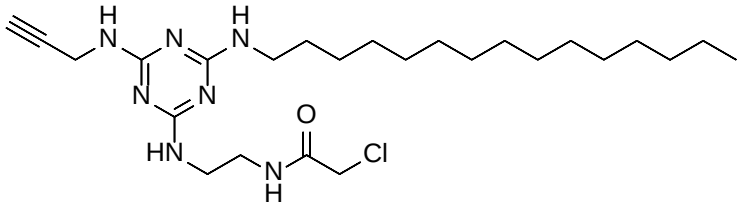
2-chloro-N-(2-((4-(dodecylamino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)acetamide (KSC-10): KSC-10-int was deprotected using a 50% solution of TFA/DCM (4 mL) at 25 °C for 2 hours. The solvent was removed *in vacuo* and the crude product remained on the vacuum overnight to remove any excess TFA. Then, to an ice cold solution of deprotected amine (1.0 eq, 24.6 mg, 0.0656 mmol) and triethylamine (2.0 eq, 23.3 mg, 0.1310 mmol) in DCM (0.01 M, 5 mL), chloroacetyl chloride (2.0 eq, 14.8 mg, 0.1310 mmol) in DCM (2 mL) was added dropwise over 30 min. The reaction was stirred at 0 °C for 3 hours. After the

reaction was complete, the solvent was removed *in vacuo* and the crude product was purified by flash chromatography to give the desired product **KSC-10** (19.1 mg, 64%). ^{1}H NMR (600 MHz, Chloroform-*d*) δ 4.28 – 4.16 (m, 2H), 4.09 (s, 2H), 3.72 – 3.48 (m, 4H), 3.47 – 3.27 (m, 2H), 2.39 – 2.16 (m, 1H), 1.70 – 1.46 (m, 2H), 1.40 – 1.06 (m, 18H), 0.87 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 167.40, 163.20, 156.28, 155.49, 117.09, 115.16, 72.23, 72.05, 71.93, 71.84, 42.35, 40.88, 39.93, 31.81, 31.09, 30.28, 29.53, 29.25, 28.88, 26.59, 22.59, 14.01. HRMS for KSC-10: m/z calcd. 452.2826; obsd. 452.2898.



KSC-11-int

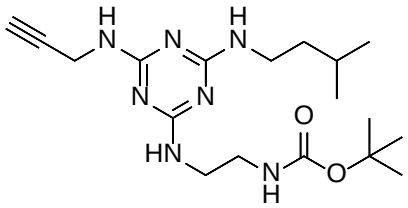
***tert*-butyl(2-((4-(pentadecylamino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)carbamate (KSC-11-int):** To a solution of **BBII** (1.0 eq, 50.9 mg, 0.1560 mmol) and diisopropylethylamine (2.5 eq, 50.9 mg, 0.3890 mmol) in THF (0.03 M, 5 mL), pentadecylamine (2.5 eq, 88.6 mg, 0.3890 mmol) was added at room temperature. The solution was then allowed to heat to reflux while stirring and continued to reflux for 24 hours. After the reaction was complete, the solvent was removed *in vacuo*. The crude product was purified via flash chromatography to afford the desired to product, **KSC-11-int** as an oil (59.6 mg, 74%). ^{1}H NMR (600 MHz, Chloroform-*d*) δ 4.13 (d, *J* = 15.5 Hz, 2H), 3.43 (d, *J* = 25.5 Hz, 2H), 3.37 – 3.06 (m, 4H), 2.19 (d, *J* = 2.5 Hz, 1H), 1.58 – 1.46 (m, 2H), 1.40 (s, 9H), 1.23 (s, 26H), 0.85 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 166.78, 166.28, 165.90, 156.52, 81.35, 79.36, 71.13, 41.73, 41.00, 40.80, 38.48, 32.21, 30.56, 30.08, 29.98, 29.94, 29.91, 29.83, 29.70, 29.65, 29.54, 28.70, 27.28, 27.15, 22.97, 22.85, 14.40, 1.29. HRMS for KSC-11-int: m/z calcd. 518.4104; obsd. 518.4182.



KSC-11

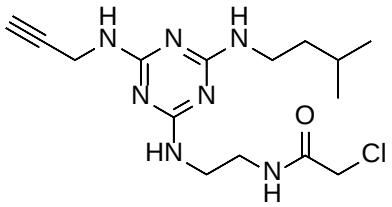
2-chloro-N-(2-((4-(pentadecylamino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)acetamide (KSC-11): KSC-11-int was deprotected using a 50% solution of TFA/DCM (4 mL) at 25 °C for 2 hours. The solvent was removed *in vacuo* and the crude product remained on the vacuum overnight to remove any excess TFA. Then, to an ice cold solution of

deprotected amine (1.0 eq, 20.3 mg, 0.0487 mmol) and triethylamine (2.0 eq, 9.9 mg, 0.0973 mmol) in DCM (0.01 M, 5 mL), chloroacetyl chloride (2.0 eq, 11.0 mg, 0.0973 mmol) in DCM (2 mL) was added dropwise over 30 min. The reaction was stirred at 0 °C for 3 hours. After the reaction was complete, the solvent was removed *in vacuo* and the crude product was purified by flash chromatography to give the desired product **KSC-11** (17.6 mg, 73%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 4.21 (s, 2H), 4.04 (d, *J* = 3.8 Hz, 2H), 3.70 – 3.24 (m, 6H), 2.24 (s, 1H), 1.56 (s, 2H), 1.25 (s, 24H), 0.87 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 170.10, 166.38, 158.26, 119.83, 117.90, 81.15, 79.88, 79.67, 79.45, 74.96, 74.67, 45.09, 44.19, 43.60, 42.65, 41.97, 34.56, 33.82, 33.00, 32.33, 31.99, 31.62, 29.32, 25.32, 16.74. HRMS for KSC-11: m/z calcd. 494.3296; obsd. 494.3394.



KSC-12-int

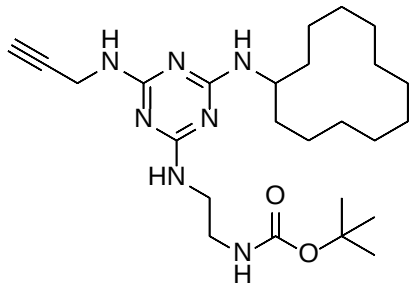
***tert*-butyl(2-((4-(isopentylamino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)carbamate (KSC-12-int):** To a solution of **BBII** (1.0 eq, 52.0 mg, 0.1590 mmol) and diisopropylethylamine (2.5 eq, 51.0 mg, 0.3980 mmol) in THF (0.03 M, 5 mL), isoamylamine (2.5 eq, 35.0 mg, 0.3980 mmol) was added dropwise at room temperature. The solution was then allowed to heat to reflux while stirring and continued to reflux for 24 hours. After the reaction was complete, the solvent was removed *in vacuo*. The crude product was purified via flash chromatography to afford the desired to product, **KSC-12-int** as an oil (49.5 mg, 83%). ^{1}H NMR (600 MHz, Chloroform-*d*) δ 4.16 (s, 2H), 3.58 – 3.17 (m, 6H), 2.20 (t, *J* = 2.5 Hz, 1H), 1.63 (dq, *J* = 13.5, 6.7 Hz, 1H), 1.41 (s, 11H), 0.91 (d, *J* = 6.6 Hz, 6H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 166.00, 156.34, 80.99, 79.31, 71.01, 41.60, 40.72, 39.02, 38.85, 30.48, 28.54, 25.81, 22.71, 22.65. HRMS for KSC-12-int: m/z calcd. 378.2539; obsd. 378.2608.



KSC-12

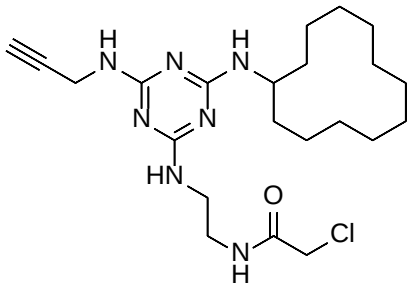
2-chloro-N-(2-((4-(isopentylamino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)acetamide (KSC-12): KSC-12-int was deprotected using a 50% solution of TFA/DCM (4 mL) at 25 °C for 2 hours. The solvent was removed *in vacuo* and the crude product

remained on the vacuum overnight to remove any excess TFA. Then, to an ice cold solution of deprotected amine (1.0 eq, 36.4 mg, 0.1310 mmol) and triethylamine (2.0 eq, 26.5 mg, 0.2620 mmol) in DCM (0.025 M, 5 mL), chloroacetyl chloride (2.0 eq, 29.5 mg, 0.2620 mmol) in DCM (2 mL) was added dropwise over 30 min. The reaction was stirred at 0 °C for 3 hours. After the reaction was complete, the solvent was removed *in vacuo* and the crude product was purified by flash chromatography to give the desired product **KSC-12** (18.2 mg, 40%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 4.18 m, 2H), 4.05 (s, 2H), 3.56 (s, 2H), 3.52 (m, 1H), 3.46 (s, 2H), 2.27 (t, *J* = 10.7 Hz, 1H), 1.64 (s, 2H), 1.55 – 1.37 (m, 2H), 0.94 (t, *J* = 8.1 Hz, 6H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 183.42, 166.94, 163.75, 115.53, 114.73, 42.76, 40.04, 39.56, 37.95, 31.25, 30.54, 30.30, 29.85, 25.82, 22.53. HRMS for KSC-12: m/z calcd. 354.1731; obsd. 354.1791.



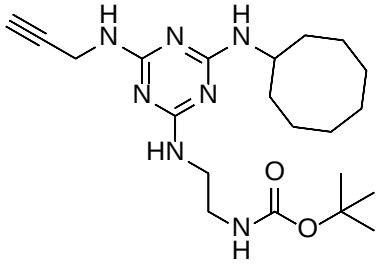
KSC-13-int

***tert*-butyl(2-((4-(cyclododecylamino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)carbamate (KSC-13-int):** To a solution of **BBII** (1.0 eq, 193.3 mg, 0.5915 mmol) and diisopropylethylamine (4.0 eq, 305.8 mg, 2.3661 mmol) in THF (0.1 M, 5 mL), cyclododecylamine hydrochloride (4.0 eq, 433.8 mg, 2.3661 mmol) was added at room temperature. The solution was then allowed to heat to reflux while stirring and continued to reflux for 24 hours. After the reaction was complete, the solvent was removed *in vacuo*. The crude product was purified via flash chromatography to afford the desired to product, **KSC-13-int** as an oil (136.6 mg, 49%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 4.25 – 4.08 (m, 2H), 3.46 (s, 2H), 3.28 (s, 2H), 2.20 (t, *J* = 2.4 Hz, 1H), 1.86 (m, 2H), 1.71 – 1.62 (m, 2H), 1.55 (m, 18H), 1.41 (d, *J* = 2.5 Hz, 9H), 1.29 – 1.21 (m, 1H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 166.29, 165.61, 156.26, 81.32, 79.04, 70.90, 55.66, 47.69, 46.07, 41.47, 40.41, 33.27, 31.17, 30.26, 29.36, 28.41, 24.30, 23.80, 23.60, 23.46, 21.88, 21.39, 18.45. HRMS for KSC-13-int: m/z calcd. 474.3478; obsd. 474.3557.



KSC-13

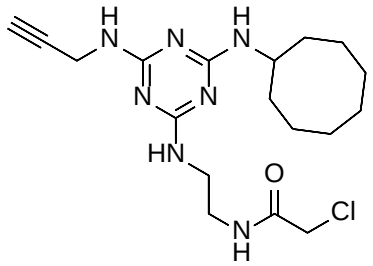
2-chloro-N-(2-((4-(cyclododecylamino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)acetamide (KSC-13): KSC-13-int was deprotected using a 50% solution of TFA/DCM (4 mL) at 25 °C for 2 hours. The solvent was removed *in vacuo* and the crude product remained on the vacuum overnight to remove any excess TFA. Then, to an ice cold solution of deprotected amine (1.0 eq, 107.7 mg, 0.2883 mmol) and triethylamine (1.3 eq, 37.9 mg, 0.3748 mmol) in DCM (0.06 M, 5 mL), chloroacetyl chloride (1.3 eq, 42.3 mg, 0.3748 mmol) in DCM (2 mL) was added dropwise over 30 min. The reaction was stirred at 0 °C for 3 hours. After the reaction was complete, the solvent was removed *in vacuo* and the crude product was purified by flash chromatography to give the desired product **KSC-13** (36.0 mg, 28%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 4.32 – 4.15 (m, 3H), 4.07 (s, 2H), 3.77 – 3.42 (m, 4H), 2.04 (t, 1H), 1.59 – 1.05 (m, 22H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 163.04, 155.11, 124.89, 117.45, 115.15, 78.69, 72.14, 60.57, 47.78, 42.53, 39.88, 30.88, 30.63, 30.37, 29.85, 24.01, 23.76, 23.38, 23.08, 21.89, 21.68, 14.33. HRMS for KSC-13: m/z calcd. 450.2670; obsd. 450.2748.



KSC-14-int

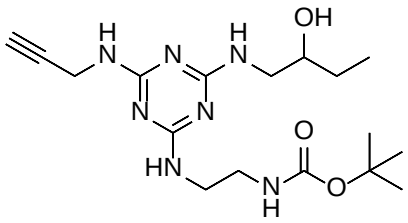
***tert*-butyl(2-((4-(cyclooctylamino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)carbamate (KSC-14-int):** To a solution of **BBII** (1.0 eq, 188.5 mg, 0.5768 mmol) and diisopropylethylamine (4.0 eq, 298.2 mg, 2.3073 mmol) in THF (0.06 M, 10 mL), cyclooctylamine (4.0 eq, 293.6 mg, 2.3073 mmol) was added dropwise at room temperature. The solution was then allowed to heat to reflux while stirring and continued to reflux for 24 hours. After the reaction was complete, the solvent was removed *in vacuo*. The crude product was purified via flash chromatography to afford the desired to product, **KSC-14-int** as an oil (158.3 mg, 66%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 4.12 (s, 2H), 3.44 (s, 2H), 3.24 (s, 2H), 2.17 (d, *J* = 2.6 Hz, 1H), 1.87 – 1.73 (m, 2H), 1.56 – 1.42 (m, 13H), 1.36 (s, 9H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ

166.37, 165.62, 164.90, 156.24, 81.22, 79.02, 70.88, 50.04, 41.56, 40.42, 32.48, 30.27, 28.40, 27.19, 25.83, 23.87. HRMS for KSC-14-int: m/z calcd. 418.2852; obsd. 418.2931.



KSC-14

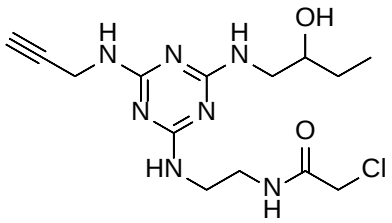
2-chloro-N-(2-((4-(cyclooctylamino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)acetamide (KSC-14): KSC-14-int was deprotected using a 50% solution of TFA/DCM (4 mL) at 25 °C for 2 hours. The solvent was removed *in vacuo* and the crude product remained on the vacuum overnight to remove any excess TFA. Then, to an ice cold solution of deprotected amine (1.0 eq, 120.3 mg, 0.3791 mmol) and triethylamine (1.3 eq, 49.9 mg, 0.4927 mmol) in DCM (0.08 M, 5 mL), chloroacetyl chloride (1.3 eq, 55.6 mg, 0.4927 mmol) in DCM (2 mL) was added dropwise over 30 min. The reaction was stirred at 0 °C for 3 hours. After the reaction was complete, the solvent was removed *in vacuo* and the crude product was purified by flash chromatography to give the desired product **KSC-14** (47.6 mg, 31%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 4.24 – 3.99 (m, 4H), 3.71 – 3.49 (m, 4H), 2.57 – 2.53 (m, 1H), 2.44 (s, 1H), 1.76 – 1.44 (m, 14H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 163.62, 144.81, 142.28, 129.95, 128.43, 125.89, 70.64, 51.58, 39.68, 35.79, 32.27, 30.85, 29.84, 28.84, 27.02, 25.13, 23.87, 21.76. HRMS for KSC-14: m/z calcd. 394.2044; obsd. 394.2122.



KSC-23-int

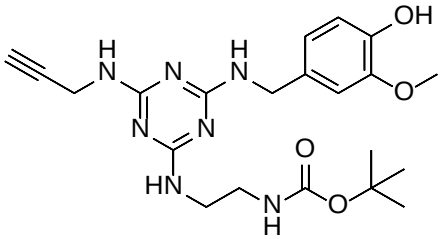
***tert*-butyl(2-((4-((2-hydroxybutyl)amino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)carbamate (KSC-23-int):** To a solution of **BBII** (1.0 eq, 53.4 mg, 0.1634 mmol) and diisopropylethylamine (4.0 eq, 84.5 mg, 0.6536 mmol) in THF (0.03 M, 5 mL), racemic 1-aminobutan-2-ol (4.0 eq, 58.3 mg, 0.6536 mmol) was added dropwise at room temperature. The solution was then allowed to heat to reflux while stirring and continued to reflux for 24 hours. After the reaction was complete, the solvent was removed *in vacuo*. The crude product was purified via flash chromatography to afford the desired to product, **KSC-23-int** as an oil (48.8 mg, 79%). ^{1}H

NMR (600 MHz, Chloroform-*d*) δ 5.57 (m, 2H), 4.08 (dd, *J* = 13.1, 6.6 Hz, 2H), 3.50 (m, 3H), 3.30 – 2.92 (m, 3H), 2.18 (d, *J* = 2.6 Hz, 1H), 1.48 – 1.39 (m, 1H), 1.36 (s, 9H), 0.90 (t, *J* = 7.6 Hz, 3H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 166.03, 165.31, 156.35, 80.79, 79.21, 73.07, 70.98, 60.42, 46.53, 40.65, 30.38, 28.42, 27.78, 14.20, 10.06. HRMS for KSC-23-int: m/z calcd. 380.2332; obsd. 380.2419.



KSC-23

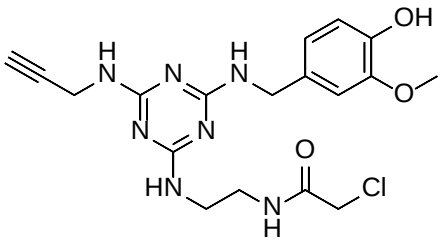
2-chloro-N-(2-((4-((2-hydroxybutyl)amino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)acetamide (KSC-23): KSC-23-int was deprotected using a 50% solution of TFA/DCM (4 mL) at 25 °C for 2 hours. The solvent was removed *in vacuo* and the crude product remained on the vacuum overnight to remove any excess TFA. Then, to an ice cold solution of deprotected amine (1.0 eq, 45.6 mg, 0.1632 mmol) and triethylamine (2.0 eq, 32.3 mg, 0.3200 mmol) in DCM (0.04 M, 4 mL), chloroacetyl chloride (2.0 eq, 36.1 mg, 0.3200 mmol) in DCM (2 mL) was added dropwise over 30 min. The reaction was stirred at 0 °C for 3 hours. After the reaction was complete, the solvent was removed *in vacuo* and the crude product was purified by flash chromatography to give the desired product **KSC-23** (23.4 mg, 40%). ^{1}H NMR (600 MHz, Chloroform-*d*) δ 4.28 – 4.10 (m, 2H), 4.06 (s, 2H), 3.98 – 3.69 (m, 1H), 3.68 – 3.48 (m, 4H), 2.38 – 2.20 (m, 1H), 1.76 (ddq, *J* = 22.3, 15.2, 7.5 Hz, 2H), 1.34 – 1.14 (m, 3H), 0.98 (dt, *J* = 20.8, 8.1 Hz, 3H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 171.18, 167.42, 163.46, 156.28, 117.10, 115.17, 113.45, 72.14, 60.34, 42.33, 29.61, 24.38, 14.09, 9.14. HRMS for KSC-23: m/z calcd. 356.1524; obsd. 356.1601.



KSC-24-int

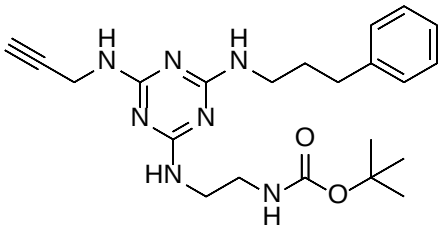
***tert*-butyl(2-((4-((4-hydroxy-3-methoxybenzyl)amino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)carbamate (KSC-24-int):** To a solution of **BBII** (1.0 eq, 132.8 mg, 0.4064 mmol) and diisopropylethylamine (2.0 eq, 105.0 mg, 0.8128 mmol) in THF (0.08 M, 5 mL),

vanillylamine hydrochloride (2.0 eq, 124.5 mg, 0.8128 mmol) was added at room temperature. The solution was then allowed to heat to reflux while stirring and continued to reflux for 24 hours. After the reaction was complete, the solvent was removed *in vacuo*. The crude product was purified via flash chromatography to afford the desired to product, **KSC-24-int** as a solid (61.7 mg, 34%). ^{1}H NMR (600 MHz, Chloroform-*d*) δ 6.84 – 6.79 (m, 1H), 6.77 (s, 2H), 4.44 (s, 2H), 4.13 (s, 2H), 3.75 (s, 3H), 3.45 (s, 2H), 3.25 (s, 2H), 2.18 (s, 1H), 1.41 (s, 9H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 165.49, 156.10, 146.77, 144.92, 130.74, 120.53, 114.72, 110.53, 79.12, 70.87, 60.29, 55.77, 44.52, 41.31, 40.51, 30.24, 29.58, 28.29, 14.09. HRMS for KSC-24-int: m/z calcd. 444.2281; obsd. 444.2364.



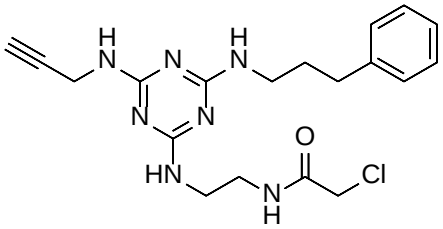
KSC-24

2-chloro-N-(2-((4-((4-hydroxy-3-methoxybenzyl)amino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)acetamide (KSC-24): KSC-24-int was deprotected using a 50% solution of TFA/DCM (4 mL) at 25 °C for 2 hours. The solvent was removed *in vacuo* and the crude product remained on the vacuum overnight to remove any excess TFA. Then, to an ice cold solution of deprotected amine (1.0 eq, 29.7 mg, 0.0865 mmol) and triethylamine (1.5 eq, 13.1 mg, 0.1298 mmol) in DCM (0.04 M, 2 mL), chloroacetyl chloride (1.2 eq, 11.7 mg, 0.1040 mmol) in DCM (2 mL) was added dropwise over 30 min. The reaction was stirred at 0 °C for 3 hours. After the reaction was complete, the solvent was removed *in vacuo* and the crude product was purified by flash chromatography to give the desired product **KSC-24** (8.0 mg, 22%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 6.92 (s, 1H), 6.71 (d, *J* = 21.5 Hz, 2H), 4.34 (s, 2H), 4.02 (m, 3H), 3.73 (s, 4H), 3.26 – 2.97 (m, 4H), 2.08 (s, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 166.04, 147.27, 145.27, 119.64, 115.09, 111.80, 55.57, 42.63, 33.64, 31.27, 30.68, 29.34, 28.99, 28.71, 28.52, 24.47, 22.07, 13.94. HRMS for KSC-24: m/z calcd. 420.1473; obsd. 420.1543.



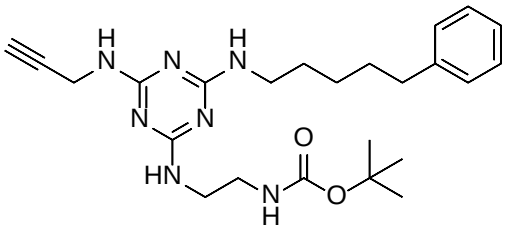
KSC-25-int

***tert*-butyl(2-((4-((3-phenylpropyl)amino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)carbamate (KSC-25-int):** To a solution of **BBII** (1.0 eq, 130.3 mg, 0.3999 mmol) and diisopropylethylamine (2.0 eq, 103.4 mg, 0.7999 mmol) in THF (0.08 M, 5 mL), 3-phenyl-1-propylamine (2.0 eq, 108.2 mg, 0.7999 mmol) was added dropwise at room temperature. The solution was then allowed to heat to reflux while stirring and continued to reflux for 24 hours. After the reaction was complete, the solvent was removed *in vacuo*. The crude product was purified via flash chromatography to afford the desired to product, **KSC-25-int** as a solid (72.6 mg, 43%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.29 – 7.23 (m, 2H), 7.19 – 7.13 (m, 3H), 4.24 – 4.06 (m, 2H), 3.52 – 3.40 (m, 2H), 3.40 – 3.31 (m, 4H), 3.27 (s, 2H), 2.65 (t, *J* = 7.8 Hz, 2H), 2.20 (t, *J* = 2.5 Hz, 1H), 1.41 (s, 9H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 166.13, 165.65, 156.29, 141.73, 128.46, 128.42, 125.92, 81.10, 79.20, 70.96, 41.54, 40.60, 40.27, 33.24, 31.44, 30.97, 30.35, 28.49. HRMS for KSC-25-int: m/z calcd. 426.2539; obsd. 426.2617.



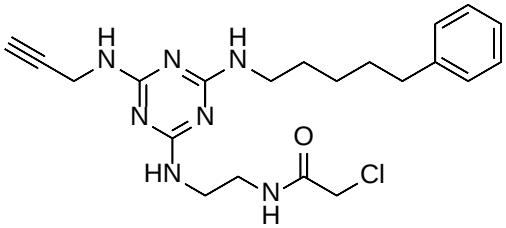
KSC-25

2-chloro-N-(2-((4-((3-phenylpropyl)amino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)acetamide (KSC-25): KSC-25-int was deprotected using a 50% solution of TFA/DCM (4 mL) at 25 °C for 2 hours. The solvent was removed *in vacuo* and the crude product remained on the vacuum overnight to remove any excess TFA. Then, to an ice cold solution of deprotected amine (1.0 eq, 55.5 mg, 0.1705 mmol) and triethylamine (3.0 eq, 51.8 mg, 0.5116 mmol) in DCM (0.08 M, 2 mL), chloroacetyl chloride (3.0 eq, 57.8 mg, 0.5116 mmol) in DCM (2 mL) was added dropwise over 30 min. The reaction was stirred at 0 °C for 3 hours. After the reaction was complete, the solvent was removed *in vacuo* and the crude product was purified by flash chromatography to give the desired product **KSC-25** (36.9 mg, 54%). ^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.26 (d, *J* = 5.7, 3.9 Hz, 2H), 7.17 (t, *J* = 5.8 Hz, 3H), 4.25 – 4.08 (m, 2H), 4.07 (s, 2H), 3.54 (s, 2H), 3.49 (s, 2H), 3.46 – 3.32 (m, 2H), 2.77 – 2.56 (m, 2H), 2.28 – 2.18 (m, 1H), 1.91 (dt, *J* = 25.9, 7.4 Hz, 2H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 163.23, 156.22, 141.12, 128.50, 126.15, 117.25, 115.34, 78.63, 72.26, 42.41, 40.48, 40.24, 32.95, 31.15, 30.29, 29.83, 8.40. HRMS for KSC-25: m/z calcd. 402.1731; obsd. 402.1809.



KSC-26-int

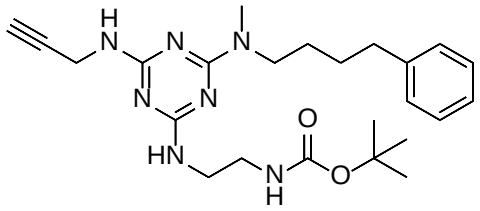
***tert*-butyl(2-((4-((5-phenylpentyl)amino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)carbamate (KSC-26-int):** To a solution of **BBII** (1.0 eq, 57.0 mg, 0.1744 mmol) and diisopropylethylamine (2.0 eq, 45.1 mg, 0.3489 mmol) in THF (0.03 M, 5 mL), 5-phenyl-1-pentylamine (2.0 eq, 57.0 mg, 0.3489 mmol) was added at room temperature. The solution was then allowed to heat to reflux while stirring and continued to reflux for 24 hours. After the reaction was complete, the solvent was removed *in vacuo*. The crude product was purified via flash chromatography to afford the desired to product, **KSC-26-int** as a solid (47.3 mg, 60%). ^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.26 (p, *J* = 5.2, 4.0 Hz, 2H), 7.16 (d, *J* = 7.9 Hz, 3H), 4.29 – 4.02 (m, 2H), 3.45 (s, 2H), 3.30 (d, *J* = 31.8 Hz, 4H), 2.66 – 2.50 (m, 2H), 2.18 (d, *J* = 20.0 Hz, 1H), 1.71 – 1.50 (m, 4H), 1.41 (d, *J* = 6.0 Hz, 11H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 166.08, 156.31, 142.60, 128.47, 128.38, 125.77, 81.05, 79.26, 70.99, 41.58, 40.69, 35.96, 31.28, 30.43, 29.76, 28.53, 26.67, 22.75, 21.16, 14.31. HRMS for KSC-26-int: m/z calcd. 454.2852; obsd. 454.2932.



KSC-26

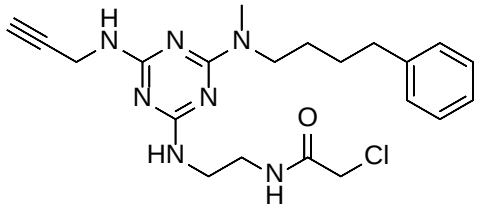
2-chloro-N-(2-((4-((5-phenylpentyl)amino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)acetamide (KSC-26): KSC-26-int was deprotected using a 50% solution of TFA/DCM (4 mL) at 25 °C for 2 hours. The solvent was removed *in vacuo* and the crude product remained on the vacuum overnight to remove any excess TFA. Then, to an ice cold solution of deprotected amine (1.0 eq, 36.2 mg, 0.1024 mmol) and triethylamine (4.0 eq, 46.2 mg, 0.4096 mmol) in DCM (0.05 M, 2 mL), chloroacetyl chloride (4.0 eq, 41.4 mg, 0.4096 mmol) in DCM (2 mL) was added dropwise over 30 min. The reaction was stirred at 0 °C for 3 hours. After the reaction was complete, the solvent was removed *in vacuo* and the crude product was purified by flash chromatography to give the desired product **KSC-26** (18.9 mg, 43%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.29 (s, 2H), 7.22 – 7.14 (m, 3H), 4.27 – 4.17 (m, 2H), 4.05 (s, 2H), 3.66 – 3.49 (m, 4H), 3.46 – 3.30 (m, 2H), 2.62 (dt, *J* = 12.6, 6.1 Hz, 2H), 2.26 (t, *J* = 2.5 Hz, 1H), 1.64 (dd, *J* = 17.7, 9.6 Hz, 6H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 200.82, 139.88, 138.15, 128.77, 128.71,

126.15, 77.67, 77.41, 77.16, 51.65, 42.93, 36.13, 31.34, 30.11, 29.30, 26.70. HRMS for KSC-26: m/z calcd. 430.2044; obsd. 430.2122.



KSC-34-int

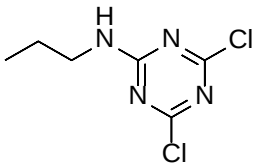
***tert*-butyl(2-((4-(methyl(4-phenylbutyl)amino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)carbamate (KSC-34-int):** To a solution of **BBII** (1.0 eq, 105.8 mg, 0.3238 mmol) and diisopropylethylamine (2.0 eq, 83.7 mg, 0.6475 mmol) in THF (0.06 M, 5 mL), N-methyl-4-phenyl-1-butylamine hydrochloride (2.0 eq, 129.3 mg, 0.6475 mmol) was added at room temperature. The solution was then allowed to heat to reflux while stirring and continued to reflux for 24 hours. After the reaction was complete, the solvent was removed *in vacuo*. The crude product was purified via flash chromatography to afford the desired to product, **KSC-34-int** as a solid (100.8 mg, 69%). ^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.31 – 7.20 (m, 2H), 7.20 – 7.11 (m, 3H), 5.27 (m, 3H), 4.11 (q, *J* = 7.1 Hz, 2H), 3.49 (d, *J* = 69.7 Hz, 4H), 3.27 (s, 2H), 3.04 (s, 3H), 2.64 (q, *J* = 6.7, 5.2 Hz, 2H), 2.24 – 2.08 (m, 1H), 1.62 (d, *J* = 7.8 Hz, 4H), 1.41 (s, 9H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 171.16, 165.19, 156.23, 142.46, 128.46, 128.33, 125.76, 79.05, 70.72, 60.43, 40.63, 35.74, 34.35, 30.37, 29.75, 28.71, 28.48, 27.10, 21.09, 14.26. HRMS for KSC-34-int: m/z calcd. 454.2852; obsd. 454.2941.



KSC-34

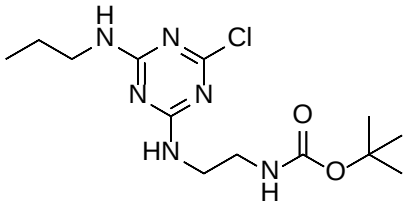
2-chloro-N-(2-((4-(methyl(4-phenylbutyl)amino)-6-(prop-2-yn-1-ylamino)-1,3,5-triazin-2-yl)amino)ethyl)acetamide (KSC-34): KSC-34-int was deprotected using a 50% solution of TFA/DCM (4 mL) at 25 °C for 2 hours. The solvent was removed *in vacuo* and the crude product remained on the vacuum overnight to remove any excess TFA. Then, to an ice cold solution of deprotected amine (1.0 eq, 100.8 mg, 0.2852 mmol) and triethylamine (1.3 eq, 37.5 mg, 0.3707 mmol) in DCM (0.15 M, 2 mL), chloroacetyl chloride (1.3 eq, 41.9 mg, 0.3707 mmol) in DCM (2 mL) was added dropwise over 30 min. The reaction was stirred at 0 °C for 3 hours. After the reaction was complete, the solvent was removed *in vacuo* and the crude product was purified by flash chromatography to give the desired product **KSC-34** (35.3 mg, 29%). ^{1}H NMR (600 MHz,

Chloroform-*d*) δ 7.28 (dt, *J* = 7.9, 4.0 Hz, 2H), 7.21 – 7.13 (m, 3H), 4.30 – 3.97 (m, 4H), 3.75 – 3.44 (m, 6H), 3.15 (d, *J* = 3.3 Hz, 3H), 2.65 (t, *J* = 7.0, 3.3 Hz, 2H), 2.10 (s, 1H), 1.74 – 1.58 (m, 4H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 162.98, 162.01, 154.53, 141.82, 128.33, 128.29, 125.85, 117.03, 115.10, 78.28, 71.44, 49.59, 49.38, 42.18, 35.51, 30.85, 30.18, 29.64, 28.43, 26.75, 26.64. HRMS for KSC-34: m/z calcd. 430.2044; obsd. 430.2102.



BBIna

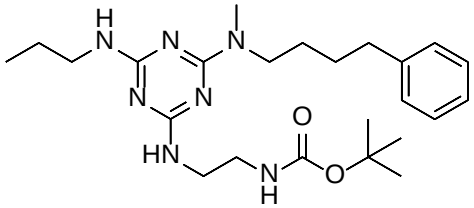
4,6-dichloro-N-propyl-1,3,5-triazin-2-amine (BBIna): To an ice cold solution of cyanuric chloride (1.0 eq, 521.0 mg, 2.8252 mmol) and diisopropylethylamine (1.1 eq, 401.6 mg, 3.1077 mmol) in tetrahydrofuran (THF) (0.043 M, 65.4 mL), propylamine (1.2 eq, 200.4 mg, 3.3903 mmol) was added dropwise. The reaction mixture was allowed to stir at 0 °C for 3 hours. The solvent was removed *in vacuo* and the crude product was purified via flash chromatography to give the desired product **BBIna** as a white solid (534.6 mg, 91%). ^{1}H NMR (600 MHz, Chloroform-*d*) δ 6.75 (d, *J* = 6.4 Hz, 1H), 3.58 – 3.20 (m, 2H), 1.63 (h, *J* = 7.4 Hz, 2H), 0.94 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 171.05, 169.64, 165.90, 43.30, 22.46, 11.26. HRMS for BBIna: m/z calcd. 207.0126; obsd. 207.0204.



BBIIna

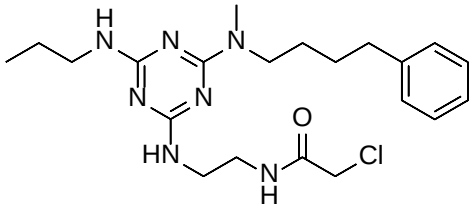
tert-butyl(2-((4-chloro-6-(propylamino)-1,3,5-triazin-2-yl)amino)ethyl)carbamate (BBIIna): To a solution of **BBIna** (1.0 eq, 534.6 mg, 2.5820 mmol) and sodium carbonate (2.2 eq, 602 mg, 5.68 mmol) in ethanol (0.09 M, 30 mL), N-Boc-ethylenediamine (1.1eq, 445 mg, 2.84 mmol) was added dropwise at room temperature. The solution was then allowed to warm to 45 °C while stirring and continued to stir for 18 hours. After the reaction was complete, 15 mL of water was added and the reaction was extracted with ethyl acetate (3 x 15 mL). The organic layer was washed with brine and dried with $MgSO_4$, then the solvent was removed *in vacuo*. The crude product was purified via flash chromatography to afford the desired to product, **BBIIna** as a white solid (695.1 mg, 81%). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 6.05 (d, *J* = 100.2 Hz, 1H), 5.80 – 5.40 (m, 1H), 5.38 – 4.72 (m, 1H), 3.64 – 3.43 (m, 2H), 3.43 – 3.24 (m, 4H), 1.69 – 1.52 (m, 2H), 1.43 (s, 9H), 1.04 – 0.85 (m, 3H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 197.31, 166.14, 156.46,

96.43, 46.67, 43.17, 41.04, 28.77, 24.63, 22.96, 11.78. HRMS for BBIIna: m/z calcd. 331.1571; obsd. 331.1649.



KSC-34na-int

tert-butyl(2-((4-(methyl(4-phenylbutyl)amino)-6-(propylamino)-1,3,5-triazin-2-yl)amino)ethyl)carbamate (KSC-34na-int): To a solution of **BBIIna** (1.0 eq, 30.2 mg, 0.0913 mmol) and diisopropylethylamine (4.0 eq, 47.2 mg, 0.3652 mmol) in THF (0.02 M, 5 mL), N-methyl-4-phenyl-1-butylamine hydrochloride (2.0 eq, 36.5 mg, 0.1826 mmol) was added at room temperature. The solution was then allowed to heat to reflux while stirring and continued to reflux for 24 hours. After the reaction was complete, the solvent was removed *in vacuo*. The crude product was purified via flash chromatography to afford the desired to product, **KSC-34na-int** as a solid (23.6 mg, 57%). ^{1}H NMR (600 MHz, Chloroform-*d*) δ 7.29 – 7.24 (m, 3H), 7.19 – 7.15 (m, 3H), 5.76 (s, 1H), 4.96 (s, 1H), 4.76 (s, 1H), 3.56 (s, 2H), 3.47 (s, 2H), 3.29 (s, 3H), 3.04 (s, 3H), 2.77 – 2.48 (m, 2H), 1.67 – 1.58 (m, 2H), 1.42 (s, 9H), 1.26 (d, *J* = 1.4 Hz, 4H), 0.94 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 157.23, 146.95, 145.10, 131.04, 130.91, 128.33, 79.86, 79.65, 79.44, 47.28, 45.14, 43.19, 38.33, 32.35, 31.32, 31.07, 29.73, 25.73, 14.16. HRMS for KSC-34na-int: m/z calcd. 458.3165; obsd. 458.32435.



KSC-34na

2-chloro-N-(2-((4-(methyl(4-phenylbutyl)amino)-6-(propylamino)-1,3,5-triazin-2-yl)amino)ethyl)acetamide (KSC-34na): KSC-34na-int was deprotected using a 50% solution of TFA/DCM (4 mL) at 25 °C for 2 hours. The solvent was removed *in vacuo* and the crude product remained on the vacuum overnight to remove any excess TFA. Then, to an ice cold solution of deprotected amine (1.0 eq, 81.8 mg, 0.2288 mmol) and triethylamine (3.0 eq, 69.5 mg, 0.6864 mmol) in DCM (0.02 M, 10 mL), chloroacetyl chloride (3.0 eq, 77.5 mg, 0.6864 mmol) in DCM (2 mL) was added dropwise over 30 min. The reaction was stirred at 0 °C for 3 hours. After the reaction was complete, the solvent was removed *in vacuo* and the crude product was purified by flash chromatography to give the desired product **KSC-34na** (95.5 mg, 96%). ^{1}H NMR (500 MHz,

Chloroform-*d*) δ 7.28 (t, *J* = 7.7 Hz, 2H), 7.20 (d, *J* = 7.3 Hz, 1H), 7.16 (t, *J* = 6.7 Hz, 2H), 3.71 – 3.47 (m, 6H), 3.39 – 3.28 (m, 2H), 3.13 (s, 3H), 2.70 – 2.58 (m, 2H), 1.74 – 1.50 (m, 6H), 1.25 (s, 2H), 0.94 (dt, *J* = 12.9, 7.5 Hz, 3H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 166.09, 141.94, 128.55, 128.43, 126.11, 78.00, 49.50, 42.75, 39.91, 39.35, 35.72, 35.64, 35.36, 29.86, 28.68, 26.95, 22.58, 11.46, 1.18. HRMS for KSC-34na: m/z calcd. 434.2357; obsd. 434.2435.

3. Experimental Methods

Covalent docking studies

Covalent docking calculations were performed by the covalent docking workflow by Schrodinger, Inc. For the calculations, the experimental structure of PDIA1 in its reduced state was used (PDB ID: 4EKZ(Wang et al., 2013)). A two-dimensional structure was prepared in ChemDraw (Perkin-Elmer) and then converted to 3D and energy minimized with default settings with LigPrep (Schrodinger). Prior to calculations, RB-11-ca was manually positioned in the **a** domain active site of PDIA1 near the catalytic cysteine, for use in automatic grid determination. The ligand was then docked using a nucleophilic substitution reaction at position C53 with default pose-prediction settings.

Cell culture and preparation of MCF-7 cell lysates

MCF-7 cells were cultured at 37 °C under an atmosphere of 5% CO_2 in RPMI 1640 media (Corning) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals) and 25 µg/mL of Amphotericin B, 10,000 units/mL of penicillin, and 10,000 µg/mL streptomycin (Gibco Anti-Anti). The cells were then harvested and the pellets washed with phosphate buffered saline (PBS). After washing, the pellets were resuspended in an appropriate amount of PBS and then sonicated with an ultrasonic tip sonicator (Cole Parmer, Vernon Hills, IL). The lysates were separated by centrifugation at 45,000 rpm for 45 minutes at 4 °C to obtain soluble and insoluble lysate fractions. The soluble fraction was collected and the pellet discarded. Protein concentrations were determined using the DC Protein Assay kit (Bio-Rad, Hercules, CA).

Fluorescent Gel Analysis

For recombinant PDI labeling gels, purified protein (50 µL, 50 µg mL^{-1}) in PBS was treated with probe (1 µL of 50x stock in DMSO) for one hour. For soluble MCF-7 cell lysates (50 µL, 2 mg mL^{-1}) in PBS were treated with probe (1 µL of 50x stock in DMSO) for one hour. Rhodamine-azide (Click Chemistry Tools) was then appended to probe labeled proteins via CuAAC; Rhodamine-azide (25 µM, 100x stock in DMSO), TCEP (1 mM, 50x stock in water), TBTA (100 µM, 17x stock in t-BuOH:DMSO 4:1), and copper (II) sulfate (1 mM, 50x stock in water) were added to the cell lysate. Samples were then incubated at room temperature for one hour to allow for the cycloaddition reaction to occur. An equal volume of SDS-PAGE loading buffer (2x, reducing) was added to each reaction and 20 µL of this solution was separated on a 10% SDS-PAGE gel. Gels were visualized for rhodamine fluorescence on a ChemiDoc MP imaging system (BioRad, Hercules, CA). Gels were then coomassie stained following a standard procedure and visualized on the same imager.

In situ labeling experiments

MCF-7 cells were grown to ~90% confluence in 10 cm tissue culture plates. Growth media was removed and replaced with 5 mL RPMI. Probes in an appropriate DMSO stock was then added to the media to achieve the desired labeling concentration and incubated at 37 °C under an atmosphere of 5% CO_2 for 3 hours. Cells were then harvested and were then prepared as lysates as described above.

KSC-34 cellular occupancy experiments

MCF-7 cells were labeled dose-dependently with the non-alkyne analogue, KSC-34na, and prepared as above. After normalizing protein concentrations to 2 mg/mL across samples, each sample was then treated with 5 µM KSC-34 for 1 hour at room temperature. Samples were then subjected to CuAAC to append a fluorophore for fluorescent-gel analysis. Gels were visualized for rhodamine fluorescence on a ChemiDoc MP imaging system (BioRad, Hercules, CA). Gels were then coomassie stained following a standard procedure and visualized on the same imager. ImageJ was used to quantify the loss of fluorescence intensity of the PDIA1 band and an EC_{50} value for cellular occupancy was determined using PRISM.

PDIA1 active site selectivity experiments

In order to determine the active site preference for each probe, mutant recombinant PDIA1, C53A or C397A (50 µg/mL), was spiked into MCF-7 lysate (1 mg/mL) followed by dose-dependent increases of KSC-34, RB-11-ca or 16F16. Following incubation, samples were then incubated with Chloroacetamide-Rhodamine (8 µM, Figure SI4), to append a fluorophore to any unlabeled PDIA1. ImageJ was used to quantify the loss of fluorescence intensity of the PDIA1 band and IC_{50} values for each active site were determined using PRISM.

PDI isoform selectivity experiments

In order to determine the selectivity of KSC-34 for PDIA1 over other PDI family members, an in-gel fluorescence experiment was performed comparing labeling of PDIA1 with KSC-34 to PDIA3 and PDIA4. The PDIs (50 µg/mL) in PBS either in the absence or presence of an MCF-7 lysate background (1 mg/mL) were treated with KSC-34 (5 µM) for one hour at room temperature. Following treatment, rhodamine-azide was appended with CuAAC for fluorescent-gel analysis. Gels were visualized for rhodamine fluorescence on a ChemiDoc MP imaging system (BioRad, Hercules, CA). Gels were then coomassie stained following a standard procedure and visualized on the same imager.

PDIA1 Insulin Reduction Activity Assay

PDIA1 WT (5 µM) in PBS was aliquoted as 25 µL samples. Inhibitor or DMSO (0.5 µL of 50x) were added to each sample to achieve a final concentration between 0 µM and 1,500 µM for

either 5, 15, 30, 45, or 60 minutes. The samples were briefly vortexed and then 10 µL was taken from each sample and added to the assay plate. Fresh assay buffer (100 mM K2HPO4, 2 mM EDTA, pH 7.0) was prepared and 70 µL was aliquoted per well. Bovine insulin (Sigma-Aldrich, 10 µL, 10x, 1.6 mM) was also added to the wells. Following a brief shake in the plate reader, DTT (10 µL, 10x, 10 mM) was added to initiate the activity assay. A sample without PDIA1 and a sample without DTT were used as blank controls. This results in final concentrations in this assay of PDIA1 WT (0.5 µM), Insulin (0.16 mM), and DTT (1 mM). The assay plate was read for absorbance at 650 nm every 20 seconds for 30 minutes, with shaking between reads.

For data analysis, each well was normalized to the – PDIA1 sample at each time point. The linear portion (400 – 800 sec) of the absorbance vs time plot was used to monitor a loss in PDIA1 activity. Linear regressions for each pre-incubation time point at each [Inhibitor] were performed to determine the rates of the reactions (5 slopes, 5 different pre-incubation times) for each [Inhibitor]. For each [Inhibitor], the Rate of Activity vs Pre-incubation time was plotted. This was determined by normalizing the reaction rates of each sample to samples without inhibitor, to give % Rate of Control vs Pre-incubation time. A one-phase decay nonlinear regression was performed for each [Inhibitor] to produce a rate constant for inhibition, k_{obs}. These rate constants were plotted as k_{obs} vs [Inhibitor], and another nonlinear regression was performed to calculate K_I and k_{inact}.

KSC-34 Mass Spectrometry Sample Preparation and Data Analysis

MCF-7 cells were grown to ~90% confluence in 15 cm tissue culture plates. Growth media was removed and replaced with 10 mL RPMI. KSC-34 (10 µL, 1000x stock) or DMSO was then added to the media to achieve the desired labeling concentration and incubated at 37 °C under an atmosphere of 5% CO_2 for 3 hours. Cells were then harvested and were then prepared as lysates as described above.

KSC-34 labeled MCF-7 cell lysates (500 µL, 2 mg mL-1) in PBS were subjected to CuAAC. Biotin azide (200 µM from 100x stock in DMSO), TCEP (1 mM, 50x stock in water), TBTA (100 µM, 17x stock in t-BuOH:DMSO 4:1), and copper (II) sulfate (1 mM, 50x stock in water) were added to the cell lysate. Samples were incubated at room temperature for 1 hour to allow for the cycloaddition reaction to occur. Samples were then centrifuged for 10 minutes at 4 °C to pellet the precipitated proteins. Protein pellets were then resuspended in cold methanol by tip sonication followed by centrifugation. Following a second methanol wash, pelleted proteins were solubilized in a 1.2% SDS/PBS solution via tip sonication and incubation at 85 °C for 5 minutes. Samples were then diluted with 5 mL PBS to lower the concentration of SDS to 0.2%. Next, samples were incubated with 100 µL streptavidin agarose beads (Thermo Fisher Scientific, Waltham, MA) at 4 °C for 16 hours. Samples were then washed with 0.2% SDS/PBS (5 mL), PBS (3 x 5 mL), and water (3 x 5 mL). The streptavidin agarose beads were pelleted between each wash step by centrifugation (1,400 g, 3 minutes).

The beads were suspended in a solution of 6 M Urea/PBS (500 µL) and 10 mM dithiothreitol (DTT, 20x stock in water), followed by incubation at 65 °C for 20 minutes. Next, iodoacetamide (20 mM, from 50x stock in water) was added to each sample and incubated at room temperature for 30 minutes. The beads were pelleted (1,400 g, 3 minutes) and resuspended in 200 µL of 2 M Urea/PBS, 1 mM CaCl2 (100x stock in water), and 2 µg trypsin (Promega, Madison, WI). On-bead trypsin digestion was allowed to proceed overnight at 37 °C with agitation.

The beads were pelleted (1,400 g, 3 min) and the supernatant collected. The beads were washed with water (2 x 50 µL) and the washes were combined with the supernatant. Formic acid (15 µL, Thermo Fisher Scientific, Waltham, MA) was then added to each sample and the samples were stored at -20 °C until mass spectrometry analysis.

LC/LC-MS/MS analysis was performed on an LTQ Orbitrap Discovery mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled to an Agilent 1200 series HPLC (Agilent Technologies, Santa Clara, CA). Tryptic digests were pressure loaded onto a 250 µm fused silica desalting column packed with 4 cm of Aqua C18 reversed phase resin (Phenomenex, Torrance, CA). Peptides were then eluted onto a biphasic 100 µm fused silica column with a 5 µm tip, packed with 10 cm of C18 and 4 cm of Partisphere SCX (Whatman, Pittsburgh, PA). Elution of the peptides from the desalting column into the biphasic column occurred using a gradient of 5-100% Buffer B in Buffer A (Buffer A: 95% water, 5% acetonitrile, 0.1% formic acid; Buffer B: 20% water, 80% acetonitrile, 0.1% formic acid). The peptides were eluted from the SCX onto the C18 resin and then into the mass spectrometer using four salt pushes (Weerapana et al., 2007). The flow rate of buffer through the fused silica column was set to 0.25 µL min^{-1} and the spray voltage was set 2.75 kV. One full MS scan (400 – 1800 MW) was followed by 8 data dependent scans of the n^{th} most intense ions with dynamic exclusion enabled.

Two biological replicates each of KSC-34 (5 µM) or DMSO treated MCF-7 cells were subjected to LC/LC-MS/MS analysis as outlined above. The generated tandem MS data was searched using the SEQUEST algorithm against the human UniProt database. A static modification of +57.0215 on cysteine was added to account for alkylation of cysteine residues with iodoacetamide. The SEQUEST output files were then filtered using DTASelect v2.0 to generate a list of proteins identified with a false-discovery rate of < 5%. The resulting peptides were then further filtered to display proteins identified in KSC-34 treated samples with an average of 10 spectral counts or greater across the biological replicates. For each of these proteins, the change in spectral counts between KSC-34 treated samples and DMSO samples was calculated and the data was ranked by those proteins displaying the highest change in spectral counts in the KSC-34 treated samples relative to the DMSO treated samples.

UPR Assays and Cell Culture

MCF7 cells were cultured in RPMI-1640 supplemented with glutamine, penicillin/streptomycin, and 10% fetal bovine serum (FBS). Cells were treated with the indicated concentrations of KSC-34 (see Results section) at the indicated time points, with 0.1% DMSO serving as negative control. To induce global ER stress, the sarco/endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin was used at 5 µM for three hours. To inhibit IRE1 activity, cells were co-treated with 4µ8C (purchased from Sigma-Aldrich) at the indicated concentrations for three hours.

Quantitative RT-PCR

The relative mRNA expression levels of target genes were measured by quantitative RT-PCR (see Table 1 for a list of primers used). Cells were lysed in tissue culture plates, and total RNA was extracted using the Omega E.Z.N.A. Total RNA Kit I according to the manufacturer's

instructions. RNA concentrations were quantified and normalized to 1 µg total RNA for cDNA reverse transcription. Using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit, cDNA was synthesized in a Bio-Rad T100 Thermal Cycler. LightCycler 480 SYBR Green I Master reaction mix (Roche), appropriate primers (purchased from Sigma-Aldrich), and cDNA were used for amplification in a LightCycler 480 Instrument II (Roche) in the MIT BioMicro Center. Primer integrity was assessed by thermal melt to ensure homogeneity. Transcripts were normalized to the housekeeping gene *RPLP2*, and all reactions were performed in quadruplicate. Experiments were repeated in biological triplicate. Data were analyzed using the ΔΔCt method and presented as mean fold change.

XBP1 Splicing Assay

cDNA synthesized from total RNA (as above) was amplified using the Q5 High-Fidelity DNA Polymerase (NEB) according to the manufacturer's instructions with the appropriate *XBP1* primers (Table 1). Five microliters of the resultant reaction were separated on 2.5% agarose gels stained with GelGreen (Biotium).

Table S1. Primers used for qPCR and XBP1 splicing assays.

Transcript	Forward	Reverse
BIP	5'- GCCTGTATTTCTAGACCTGCC-3'	5′-TTCATCTTGCCAGCCAGTTG-3′
CHOP	5′-GGAGCTGGAAGCCTGGTATG-3′	5′-GCCAGAGAAGCAGGGTCAAG-3′
ERDJ4	5'-CTGTATGCTGATTGGTAGAGTCAA-3'	5'-AGTAGACAAAGGCATCATTTCCAA-3'
GADD34	5'-TGGTAGAAGCTGGCCTGGAG-3'	5'-GGGAACTGCTGGTTTTCAGC-3'
GRP94	5'-GGCCAGTTTGGTGTCGGTTT-3'	5'-CGTTCCCCGTCCTAGAGTGTT-3'
HYOU1	5′-GCAGACCTGTTGGCACTGAG-3′	5′-TCACGATCACCGGTGTTTTC-3′
RPLP2	5′-CCATTCAGCTCACTGATAACCTTG-3′	5′-CGTCGCCTCCTACCTGCT-3′
SEC24D	5′-AGCAGACTGTCCTGGGAAGC-3′	5'-TTTGTTTGGGGCTGGAAAAG-3′
XBP1	5'-CCTTGTAGTTGAGAACCAGG-3'	5'-GGGGCTTGGTATATATGTGG-3'

Cell culture and transfections

HEK293^{TREX}, HEK293^{DAX} (Shoulders et al., 2013), and HEK293^{DAX} *PDIA1* shRNA cells were cultured in High-Glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with glutamine, penicillin/streptomycin and 10% fetal bovine serum. *PDIA1* shRNA knockdown cells were additionally cultured with 5ug/ml puromycin. HEK293^{DAX} cells were transfected with ^{FT}ALLC.pcDNA3.1 using calcium phosphate, as previously described (Cooley et al., 2014; Plate et al., 2016). All cells were cultured under typical tissue culture conditions (37°C, 5% CO2).

Cytotoxicity assays

HEK293^{TREX} and HEK293^{DAX} cells were plated at 5,000 cells/well in a poly-D-lysine coated transparent, flat-bottomed 96 well plate. Cells were pretreated for 4 hours with KSC-34 or DMSO vehicle. This media was replaced with fresh media containing KSC-34 or vehicle and conditioned for 2 hours. Cell metabolic activity was measured using the CellTiter-Glo assay (Promega).

CellTiter-Glo reagent was added to cell culture media at a 1:1 ratio and incubated for 2 min on an orbital shaker to induce cell lysis. The plate was then incubated at room temperature for 10 min to stabilize the luminescent signal and read on a Tecan F200 Pro microplate reader.

[^{35}S] Metabolic labeling experiments

HEK293^{DAX} cells plated on poly-D-lysine coated dishes were metabolically labeled in DMEM-Cys/-Met (Corning CellGro, Mediatech Inc., Manassas, VA) supplemented with glutamine, penicillin/streptomycin, 10% dialyzed fetal bovine serum, and EasyTag EXPRESS [^{35}S] Protein Labeling Mix (Perkin Elmer) for 30 min. Cells were washed twice with complete media and incubated in pre-warmed DMEM. At the indicated time, media was collected and lysates were prepared in RIPA buffer with fresh protease inhibitor cocktail (Roche). FLAG-tagged ALLC was immunopurified using M1 anti-FLAG agarose beads (Sigma Aldrich) and washed four times with RIPA buffer. The immunoisolates were then eluted by boiling in Laemmli buffer and separated on SDS-PAGE. The gels were then dried, exposed to phosphorimager plates (GE Healthcare, Pittsburgh, PA), and imaged with a Typhoon imager. Band intensities were quantified by densitometry in ImageQuant. Fraction secreted was calculated using the equation: fraction secreted = [extracellular [^{35}S]-^{FT}ALLC signal at t / (extracellular [^{35}S]- ^{FT}ALLC signal at t=0 + intracellular [^{35}S]- ^{FT}ALLC signal at t=0)].

Immunoprecipitation and SDS-PAGE

For immunoprecipitations, cells were washed with PBS then incubated with with 0.5 mM of the cell permeable reversible crosslinker Dithiobis(succinimidiyl propionate) (DSP) for 30 min at room temperature. The reaction was quenched by addition of 100 mM Tris pH 7.5. Lysates were then prepared in RIPA buffer and cleared by centrifugation at 10000 x *g* for 15 min. Proteins were immunopurified using using M1 anti-FLAG agarose beads (Sigma Aldrich). After four washes in RIPA buffer, proteins were eluted by boiling in Laemmli buffer + 100 mM DTT and samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with the following primary antibodies: monoclonal mouse M2 anti-FLAG (1:500, Sigma Aldrich), rabbit polyclonal anti-PDIA1 (1:1000, GeneTex GTX101468, Irvine, CA), rabbit polyclonal anti-PDIA4 (1:1000, Proteintech Group 14712-1-AP, Rosemont, IL).

Light chain ELISA

HEK293^{TREX} stably expressing ^{FT}ALLC or transfected HEK293^{DAX} cells were plated at 10,000 cells/well in a poly-D-lysine coated transparent, flat-bottomed 96 well plate. Triplicate wells were pretreated for 4 hours with 40µM KSC-34 or vehicle. Wells were washed twice with 100µL media and then fresh media with 40µM KSC-34 or vehicle was added for 2-hour conditioning. Conditioned media was harvested into a 96-well plate. Free LC concentrations were determined by ELISA in 96-well plates (Immulon 4HBX, Thermo Fisher). Wells were coated overnight at 37 ºC with sheep polyclonal free λ LC antibody (Bethyl Laboratories, A80-127A) at a 1:500 dilution in

50 mM sodium carbonate (pH 9.6). In between all incubation steps, the plates were rinsed extensively with Tris-buffered saline containing 0.05% Tween-20 (TBST). Plates were blocked with 5% non-fat dry milk in TBST for 1 hr at 37ºC. Media analytes were diluted between 5 – 200 fold in 5% non-fat dry milk in TBST and 100 µL of each sample was added to individual wells. Light chain standards ranging from 3 – 300 ng/mL were prepared from purified human Bence Jones λ light chain (Bethyl Laboratories, P80-127). Plates were incubated at 37 ºC for 1.5 hr while shaking. Finally, HRP-conjugated goat anti-human λ light chain antibody (Bethyl Laboratories, A80-116P) was added at a 1:5,000 in 5% non-fat dry milk in TBST, followed by a 1.5 hr incubation of the plates at 37 ºC. The detection was carried out with 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, 0.18 mg/mL) and 0.03% hydrogen peroxide in 100 mM sodium citrate pH 4.0. Detection solution (100 µL) was added to each well and the plates were incubated at room temperature. The absorbance was recorded at 405 nm and the values for the LC standards were fitted to a 4-parameter logistic function. Light chain concentrations were averaged from 3 independent replicates under each treatment and then normalized to vehicle conditions.

Quantitative RT-PCR

Cells were treated as described at 37°C, washed with Dulbecco's phosphate-buffered saline, and then RNA was extracted using the RNeasy Mini Kit (Qiagen). qPCR reactions were performed on cDNA prepared from 500 ng of total cellular RNA using the QuantiTect Reverse Transcription Kit (Qiagen). The FastStart Universal SYBR Green Master Mix (Roche), cDNA, and appropriate human primers for *PDIA1 (*TCTTCATCGACAGCGACCAC and ATCCTCTCTGCCGTCAGCTC) purchased from Integrated DNA Technologies were used for amplifications (45 cycles of 1 min at 95°C, 10 s at 95°C, 30 sec at 60°C) in an ABI 7900HT Fast Real Time PCR machine. Transcripts were normalized to the housekeeping gene *GAPDH* and all measurements were performed in triplicate. Data were analyzed using the RQ Manager and DataAssist 2.0 softwares (ABI, Foster City, CA).

Table S2. Mass spectrometry data for KSC-34 treated MCF-7 cells. The proteins shown are sorted by the spectral count difference between the KSC-34 treated and the DMSO treated samples.

Protein	UniProt ID	DMSO_1	DMSO_2	Average DMSO	KSC-34_5uM_1	KSC-34_5uM_2	Average 5 uM KSC-34	Change in Spectral Count (KSC-34 - DMSO)
P4HB	P07237	9	0	5	1704	2355	2030	2025
PTGES2	Q9H7Z7	0	0	0	94	150	122	122
FAM213A	Q9BRX8	0	0	0	70	143	107	107
HMOX2	P30519	0	0	0	57	75	66	66
HSP90AA1	P07900	31	13	22	63	87	75	53
HSP90AB1	P08238	40	23	32	72	96	84	53
TUBB	P07437	62	82	72	89	154	122	50
ACTB	P60709	215	144	180	190	247	219	39
PKM	P14618	48	46	47	59	113	86	39
TUBB4B	P68371	63	92	78	89	142	116	38
ATP2A2	P16615	5	2	4	34	48	41	38
EEF2	P13639	21	15	18	35	69	52	34
TUBB2B	Q9BVA1	49	64	57	67	110	89	32
SELT	P62341	0	0	0	19	45	32	32
HMGB1	P09429	0	8	4	18	46	32	28
RTN3	O95197	0	0	0	20	34	27	27
FASN	P49327	64	43	54	57	101	79	26
ALDOA	P04075	19	14	17	34	49	42	25
VDAC2	P45880	2	0	1	16	30	23	22
TKT	P29401	8	8	8	24	33	29	21
PDIA6	Q15084	3	3	3	13	33	23	20
TXNDC12	O95881	0	0	0	21	17	19	19
TUBA8	Q9NY65	62	0	31	31	66	49	18
G6PD	P11413	3	2	3	15	25	20	18
TXNRD1	Q16881	0	2	1	10	26	18	17
TUBB3	Q13509	44	76	60	62	90	76	16
EEF1A1	P68104	41	29	35	44	56	50	15
RTN4	Q9NQC3	0	0	0	10	20	15	15
ACTA1	P68133	52	55	54	78	58	68	15
HSPA8	P11142	22	19	21	32	36	34	14
GMPS	P49915	0	0	0	13	14	14	14
FLNA	P21333	67	56	62	63	86	75	13
CFL1	P23528	3	20	12	30	18	24	13
TPI1	P60174	5	10	8	14	26	20	13
SORD	Q00796	0	2	1	14	12	13	12
PRKDC	P78527	7	6	7	14	23	19	12
CYB5B	O43169	0	0	0	5	16	11	11
PGK1	P00558	8	10	9	17	21	19	10
ASAH1	Q13510	0	0	0	9	11	10	10
RPL4	P36578	11	5	8	13	23	18	10
RPL3	P39023	4	0	2	9	15	12	10
PGAM1	P18669	0	0	0	10	9	10	10
ACTBL2	Q562R1	17	0	9	15	21	18	10
LGALS3	P17931	0	0	0	7	12	10	10
GPI	P06744	4	5	5	12	15	14	9

Acknowledgements

This work was supported in part by the National Institute of Environmental Health Sciences of the NIH under award P30-ES002109.

References:

Cooley, C.B., Ryno, L.M., Plate, L., Morgan, G.J., Hulleman, J.D., Kelly, J.W., and Wiseman, R.L. (2014). Unfolded protein response activation reduces secretion and extracellular aggregation of amyloidogenic immunoglobulin light chain. Proc Natl Acad Sci U S A *111*, 13046-13051.

Plate L, Cooley CB, Chen JJ, Paxman RJ, Gallagher CM, Madoux F, Genereux JC, Dobbs W, Garza D, Spicer TP, Scampavia L, Brown SJ, Rosen H, Powers ET, Walter P, Hodder P, Wiseman RL, Kelly JW. (2016) Small molecule proteostasis regulators that reprogram the ER to reduce extracellular protein aggregation. Elife. 2016 Jul 20;5. pii: e15550.

Wang, C., Li, W., Ren, J., Fang, J., Ke, H., Gong, W., Feng, W., and Wang, C.C. (2013). Structural insights into the redox-regulated dynamic conformations of human protein disulfide isomerase. Antioxid Redox Signal *19*, 36-45.

Weerapana, E., Speers, A.E., and Cravatt, B.F. (2007). Tandem orthogonal proteolysis-activity-based protein profiling (TOP-ABPP)--a general method for mapping sites of probe modification in proteomes. Nat Protoc *2*, 1414-1425.