Supporting Information for

Iterative *In situ* Click Chemistry Assembles a Branched Capture Agent and Allosteric Inhibitor for Akt1

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Results

**Figure S1: Structure of Ac7.** This compound was isolated from a library of putative ATP-competitive compounds and was found to inhibit Akt1-S473E-T308P with an IC\(_{50}\) = 90 µM.

**Figure S2: Structures of Fmoc-Azido Amino Acids Used in This Work.** The synthesis of Fmoc-Az1 and Fmoc-Az2 is described in the Supplementary Methods. The syntheses of Fmoc-Az4 and Fmoc-Az8 have been described previously\(^1\).
Figure S3: Sequences obtained from the initial anchor peptide screen. Antibodies and their dilutions are also shown (5G3 = mAb against kinase domain (CST), L32A4 = mAb against phospho-T308 (CST), 2H10 = mAb against C-terminal peptide (CST). Positions with a strong consensus residue(s) are shaded.
Figure S4: Frequency of amino acids at each position for 22 selected sequences in the initial anchor peptide screen. The frequency of amino acids at each position was tabulated and used to generate a focused library.
Figure S5: Sequences obtained from the anchor peptide screen with focused library. The number of times each sequence appeared is shown to the right. Positions with a strong consensus residue(s) are shaded. The focused library was of the form NH$_2$-Az$\bar{X}_1$-$X_2$-$X_3$-$X_4$-$X_5$-GYM-TG where $X_1 = H, E, I, Q, V, G$; $X_2 = P, F, A, G, H, T$; $X_3 = E, D, V, Y$; $X_4 = N, G, D, P, Q, R, T$; $X_5 = R, L, I, T, G, E, D$. 

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Figure S6: Inhibition of Akt1-S473E-T308P by Ac7, Anchor Peptide, and Ac7-peptide Conjugate. Kinase reactions were carried out with 120 µM of each compound for 30 minutes at room temperature. The amount of phosphorylated substrate was quantitated by liquid scintillation counting and the % Activity was determined based on the amount of product formed in the control kinase reaction. The values shown are the mean value of three experiments and the error bars are the standard deviation.
Figure S7: Anchor peptide is not phosphorylated by Akt1-S473E-T308P. Biotinylated anchor peptides were incubated in a standard kinase reaction mixture for 30 min. at room temperature. A portion of the reaction was spotted onto a SAM2 Biotin Capture Membrane (Promega), washed, and analyzed by liquid scintillation counting. The total counts per minute (cpm) are shown for the biotinylated anchor peptides VFYRL-Bio(1) and VFYRL-Bio(2) as well as the biotinylated crosstide (Bio-Crosstide), the standard substrate peptide. For the anchor peptides, the number in parentheses identifies the diastereomer based on retention time in RP-HPLC.
Figure S8: Sequences obtained from the secondary peptide screen with a naïve library.

Positions with a strong consensus residue(s) are shaded. Pra = propargylglycine.
Figure S9: 5HA-Biligand-Bio. Structure of biotinylated biligand anchor for the triligand branch screen.
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**Figure S10:** Sequences obtained from the tertiary peptide screen with a naïve library. Positions with a strong consensus residue(s) are shaded.
Figure S11: Sequences obtained from the tertiary peptide screen with a focused library. Positions with a strong consensus residue(s) are shaded. The focused library was of the form: NH$_2$-Az8-X$_1$-X$_2$-X$_3$-X$_4$-X$_5$-GYM-TG where X$_1$ = A, E, H, K, L, R; X$_2$ = A, H, K, L, R; X$_3$ = D, H, K, L, E; X$_4$ = D, H, I, K, N, R, S; X$_5$ = F, G, H, I, K.
Figure S12: Determination of $K_i'$ and inhibition mode for triligand with respect to peptide substrate. (a) Plot of $1/K_M$(app) vs. [Triligand]. $K_M$(app) values were obtained from nonlinear regression analysis of velocity vs. [Peptide] curves obtained with varied concentrations of triligand (GraphPad). The error bars represent the standard error of $K_M$(app) by nonlinear regression. 95% confidence intervals are shown as dotted lines. The X-intercept (-$K_i'$) was found to be 3.6 µM. (b) Plot of $1/V_{\text{max}}$(app) vs. [Triligand]. Values for $V_{\text{max}}$(app) and standard error were obtained by nonlinear regression as described above. The X-intercept was found to be 1.7 µM. (c) Dixon plot of $1/v$ vs. [Triligand] at various [Peptide]. Data from low peptide concentrations were removed to account for possible substrate depletion. The parallel lines are diagnostic for uncompetitive inhibition with respect to peptide. (d) Cornish-Bowden plot of [Peptide]/v vs. [Triligand] at various [Peptide]. The intersection of the lines gives the –$K_i'$ (2.6 µM). The shape of this plot is diagnostic for uncompetitive inhibition with respect to peptide.
Figure S13: Determination of $K_i$ and inhibition mode for triligand with respect to ATP. (a) Plot of $1/K_{M(app)}$ vs. [Triligand]. $K_{M(app)}$ values were obtained from nonlinear regression analysis of velocity vs. [ATP] curves obtained with varied concentrations of triligand (GraphPad). The error bars represent the standard error of $K_M(app)$ by nonlinear regression. The negative slope of the line was taken as an indication that $K_m$ was unchanged during inhibition with the triligand. (b) Plot of $1/V_{max(app)}$ vs. [Triligand]. Values for $V_{max(app)}$ and standard error were obtained by nonlinear regression as described above and 95% confidence intervals are shown as dotted lines. The X-intercept was found to be 5.8 µM with wide error range based on the 95% CI. (c) Dixon plot of $1/v$ vs. [Triligand] at various [ATP]. Data from low ATP concentrations were removed due high counting error resulting from low counts per minute (cpm). The lines converged on a common X-intercept which was used to determine $K_i$ (4 µM). The shape of the plot is consistent with noncompetitive inhibition with respect to ATP.
Figure S14: PCR of single TentaGel beads. PCR was carried out on single beads as described in the text. Analysis by agarose gel electrophoresis showed a single band at approx. 100 bp.
Figure S15: Coomassie-stained gel from immunoprecipitation experiment. (A) A representative 12% gel Stained with Coomassie and imaged. Lane 1: Lysate, Lane 2: blank resin, Lane 3: anchor resin, Lane 4: biligand resin, Lane 5: triligand resin, Lane 6: mAb resin. The band at 60 kDa is full length Akt. (B) Full gel image. The band at ~50 kDa is presumed to be GSK3β.
Figure S16: Structure of Fluorescein-triligand
Figure S17: Competition ELISA suggests antibody interference with multiligand binding site. Streptavidin agarose-immobilized anchor, biligand, and triligand were probed with varying concentrations of Akt-S73E. Binding was detected by an anti-Akt1 monoclonal antibody ([2H10]) followed by an anti-mouse secondary antibody-HRP conjugate. The fraction bound was normalized and plotted against the concentration of Akt-S473E on a log scale. The data indicates that as the multiligand size increases, the [2H10] antibody binding is reduced, suggesting that the biligand and triligand may have some binding interface overlap with antibody binding at the C-terminus of Akt1.
Figure S18: Binding of Akt1 and GSK3β to Immobilized Biligand and Triligand. (A) Biotinylated biligand and triligand were immobilized on streptavidin-coated ELISA plates and incubated with varying concentrations of Akt1-S473E or GSK3β for 2 hr at 4 °C. After washing, the wells were probed with Anti-His6-HRP antibody (Santa Cruz), washed and developed. The $A_{450}$ (net) was plotted against the kinase concentration and the curve fitted with nonlinear regression using a single-site binding model (GraphPad). (B) The dissociation constants ($K_d$) as determined by nonlinear regression. The error is the standard error of the fitted curve.
METHODS

Expression and Purification of Akt1-S473E Kinase Domain

The pET28a-PKB expression plasmid (His-ΔPH-PKB-EEE-FLAG) was obtained as a generous gift from Dr. Shoshana Klein. The sequence encoding the N-terminal His6 tag through the C-terminal FLAG tag was amplified by PCR. BamH1 and EcoR1 sites were incorporated and the 5’ and 3’ ends of the amplified fragment using the following amplification primers: AktpVL-FP (Forward Primer) 5’...AAGGAGGGATCCATGGGCAGCAGCCAT...3’ and AktpVL-RP (Reverse Primer) 5’...TGGTGTAATTCTTATCAGCTTCATCGTCATC...3’. The amplified fragment was digested with BamH1 and EcoR1, purified by agarose gel electrophoresis, and added to pVI1393 insect cell expression vector that was previously digested with BamH1 and EcoR1 and dephosphorylated. After transformation and colony screening, successful ligation products were isolated and sequenced using the standard phF and mR sequencing primers. To increase the expression level, the BamH1 site was oblated and replaced with a Kozak sequence (GCCGCCACCATG) using QuickChange Mutagenesis (Forward Primer:__5’...ACCGTCCCACCATCGGGGCGCCACCATGGGCAGCAGCCAT...3’, Reverse Primer: 5’...ATGGCTGCTGCCCATGGTGCGGCGGCCCGATGGTGGGACGGT...3’). The final construct, pVLAKT.2 was given to the Caltech Protein Expression Center for construction of the viral expression vector and expression in Hi5 insect cells according to previously described protocols.

The cell pellet was lysed at 4 °C for 15 min in MPER lysis buffer (Thermo) and centrifuged at 14,000 × g twice to remove cellular debris. The resulting lysate was passed over a 1 mL HisTrap Ni-NTA column and eluted with 10 mL buffer containing 200 mM imidazole. The fractions containing the highest protein concentration were concentrated and desalted using an Amicon Ultracel centrifugal filter device (10000 MWCO, Millipore). The resulting solution was purified by Anion Exchange chromatography as previously described. The major product was
confirmed to be Akt-S473E by SDS-PAGE and western blotting with the [2H10] Anti-Akt1 antibody (Cell Signaling Technology). A single band at 45 kDa was observed corresponding to the expected product (Predicted MW = 45860). Analysis by ESI-MS showed peaks at [M+H]+ = 45835.0 (minor) and [M+H]+ = 45992.0 (major) corresponding to unmodified Akt1-S473E (predicted [M+H]+Monisotopic = 45832.0) and diphosphorylated Akt1-S473E (predicted [M+H]+Monisotopic = 45992.0) respectively. The minor product obtained from anion-exchange chromatography was analyzed by SDS-PAGE and western blotting with the (L32A4) phospho-Akt antibody (Cell Signaling Technology) and found to be phosphorylated at Thr308 (Akt1-S473E-T308P).

**Synthetic Protocols**

Scheme S1: Synthesis of Ac7. Starting material was synthesized as described previously. 4

2-Fluoro-4-(prop-2-ynyloxy)-6-(1-methyl-1H-pyrrol-2-yl)-1,3,5-triazine (1). 2,4-difluoro-6-(1-methyl-1H-pyrrol-2-yl)-1,3,5-triazine 1 (1.5 g, 7.6 mmol) was dissolved in acetonitrile (25 ml) followed by the addition of propargyl alcohol (0.4 mL, 7.1 mmol) and diisopropylamine (1.3 ml, 7.6 mmol). The reaction mixture was stirred at room temperature for 1 h, after which a precipitate formed and was isolated by filtration. The solid was washed with hexanes and dried under vacuum to give 1 as a white solid, 1.4 g, 6.5 mmol, 85%:

1H-NMR (300MHz, DMSO-d6): δ 7.31-7.34 (m, 2H), 6.24-6.26 (m, 1H), 5.15-5.162 (d, 2H), 4.07 (s, 3H), 3.69-3.71 (t, 1H); 13C-NMR (600MHz, DMSO-d6): δ 172.21, 170.93, 169.51, 134.31,
127.43, 121.01, 109.85, 79.18, 78.31, 56.75, 38.67; HRMS (ESI+) for C₁₁H₉ON₄F [M+H⁺] calcd. 232.0760; found 232.0771

Scheme S2: Synthesis of Fmoc-Az1 and Fmoc-Az2. Az1 was synthesized as previously described with modifications⁵.

(2): Triflic anhydride, Tf₂O (3.00 ml, 17.8 mmol) was added dropwise to a vigorously stirred mixture of NaN₃ (5.76 g, 88.6 mmol) in 15 mL H₂O and 30 mL DCM at 0 °C. The resulting mixture was allowed to warm to ambient temperature and stirred for 2 h. The water layer was extracted with DCM (2 × 15 mL) and the combined organic layers were washed with saturated aqueous Na₂CO₃ solution (25 mL). Fmoc-Dap-OH (n = 1, 2.89 g, 8.86 mmol) dissolved in 80% aqueous acetic acid (26.6 mL) and CuSO₄.5H₂O (0.044 g, 0.18 mmol) in 3 mL H₂O was added. The pH of the solution was adjusted to 9-10 with saturated K₂CO₃ solution. Into a mixture of H₂O (45 mL) and methanol (95 mL), TfN₃ (6 mmol) in DCM (15 mL) was added and the pH was readjusted to 9-10 with dropwise addition of saturated K₂CO₃ solution. The two-phase system was stirred vigorously for 20 h. The layers were separated by addition of DCM and the organic layer was washed with water (2 × 40 mL) and then the combined aqueous phases were acidified with 3 M HCl to pH 2. The aqueous phase was extracted with DCM (4 × 50 mL) and the combined organic phases were dried over Na₂SO₄, filtered and concentrated in vacuum to give (2) as a white solid.
$^1$H-NMR (400MHz, DMSO-d$_6$): $\delta$ 3.60-3.63 (m, 2H), 4.20-4.27 (m, 2H), 4.30-4.35 (m, 2H), 7.32 (t, 2H, $J = 7.4$ Hz), 7.42 (t, 1H, $J = 7.4$ Hz), 7.73 (d, 2H, $J = 7.4$ Hz), 7.89 (d, 2H, $J = 7.4$ Hz), 7.93 (d, 1H, $J = 8.0$ Hz), 12.64 (s,1H); $^{13}$C-NMR (100 MHz, DMSO-d$_6$): $\delta$ 46.8, 51.0, 54.0, 66.3, 120.5, 125.7, 127.2, 128.5, 140.8, 144.6, 156.4, 171.8.

(3): Synthesis was carried out as described above, except Fmoc-Dab-OH (n = 2) was used as the starting material.

$^1$H-NMR (400MHz, DMSO-d$_6$): $\delta$ 1.80-1.88 (m, 1H), 1.92-2.02 (m, 1H), 3.31-3.38 (m, 1H), 3.41-3.47 (m, 1H), 4.00-4.06 (m, 1H), 4.23 (t, 1H, $J = 6.8$ Hz), 4.28-4.32 (m, 3H), 7.32 (t, 2H, $J = 7.4$ Hz), 7.42 (t, 2H, $J = 7.4$ Hz), 7.64 (d, 1H, $J = 7.4$ Hz), 7.70 (d, 2H, $J = 7.4$ Hz). 12.65 (s,1H); $^{13}$C-NMR (100 MHz, DMSO-d$_6$): $\delta$ 31.1, 47.1, 48.0, 51.6, 66.6, 120.6, 125.7, 127.5, 128.1, 141.2, 144.2, 156.6, 173.8.

Peptides were synthesized standard Fmoc SPPS protocols either manually or on a Titan 357 automated peptide synthesizer (AAPPTEC). Libraries were synthesized on 90 μm TentaGel S (NH$_2$) (Rapp Polymere). Biotinylated peptides were synthesized on Biotin NovaTag Resin (EMD). Side-chain protected peptides were synthesized on Sieber Amide Resin (Anaspec) while C-terminal amide peptides were synthesized on Rink Amide MBHA resin (Anaspec). The natural Fmoc-L amino acids were purchased from AAPPTEC and the Fmoc-L-propargylglycine was purchased from (Chem-Impex). The azido-amino acids Az4, and Az8 were synthesized according to previously published procedures.$^1$

Briefly, resins were swelled in NMP and deprotected with 20% piperidine. 4 equivalents of Fmoc-amino acid (natural L-amino acids and L-propargylglycine), 3.9 equivalents of HATU, and 12 equivalents of DIEA were added (equivalents relative to loading capacity of the resin). Couplings proceeded for 30-45 minutes. Azido amino acids were added at 2 equivalents relative to the resin loading capacity. The N-termini were acetylated with 20 equivalents of acetic anhydride and 10 equivalents of DIEA. In cases where use of Azido-amino acids produced a mixture of two diastereomers, the diastereomers were purified as a single product unless otherwise noted.
Synthesis of Biligand Linker Variants

For the analysis of the effect of linker length, three biligand variants were synthesized. The Anchor peptide was synthesized on 150 mg scale on Rink amide MBHA resin and appended with one of three azido amino acids with 1, 4, or 8 methylene units between the Cα carbon and side chain azide (Fmoc-Az1, Fmoc-Az4, and Fmoc-Az8, Figure S2). Following the acylation of the N-terminus with acetic anhydride, the resin was resuspended in NMP. The secondary ligand (Ac-Pra-FWFLRG-CONH₂) was synthesized on 300 mg scale on Sieber amide resin. The peptide was cleaved by adding 4.5 mL 2% TFA in CH₂Cl₂ and incubating for 5 minutes. The TFA was quenched by filtration into 225 µL DIEA. The cleavage was repeated five times, the filtrates were combined, and the solvent removed by rotary evaporation. The protected secondary peptide was the purified by C18 RP-HPLC with a dH₂O:CH₃CN (0.1% TFA) gradient.

The biligand variants were synthesized by combining 12 mg of anchor peptide on Rink MBHA resin (~ 8 µmol azide) with 24 µmol side-chain protected secondary peptide in the presence of 40 mM CuI, 60 mM L-ascorbic acid, and 20% piperidine. The reaction proceeded for 6 hr at room temperature with agitation. The copper was removed by exhaustive washing with copper chelation solution (22 mM sodium diethylthio carbamate (trihydrate), 29 mM DIEA, in DMF) followed by NMP. The biligands were cleaved from the resin in 95:5:5 TFA:H₂O:TES, precipitated in diethyl ether, and purified by C18 RP-HPLC with a dH₂O:CH₃CN (0.1% TFA) gradient. MALDI-TOF MS: n = 1: Expected [M+H]^+ = 2031.04, Observed [M+H]^+ = 2029.66. n = 4: Expected [M+H]^+ = 2073.09, Observed [M+H]^+ = 2070.05. n = 8: Expected [M+H]^+ = 2129.15, Observed [M+H]^+ = 2125.73.

Synthesis of 5HA-Biligand-Bio (Figure S9)

The biotinylated anchor peptide VFYRLGY-Bio was synthesized according to standard protocols on biotin NovaTag resin (EMD). Following addition of the Az8 residue, the resin was washed
with NMP and set aside (Fmoc-Az8-VFYRLGY-Biotin). In parallel the secondary ligand (Ac-Pra-FWFLRG-CONH₂) was synthesized on Sieber amide resin and cleaved from the resin with side-chain protecting groups intact (see above). The peptide was purified by RP-HPLC using a dH₂O:CH₃CN gradient with 0.1% TFA. The product was confirmed by MALDI-TOF.

The biligand was assembled on-resin according to the following procedure: 30 mg of resin-bound Fmoc-Az8-VFYRLGY-Biotin (14 µmol) was washed and added to 47 µmol protected secondary peptide (Ac-Pra-FWFLRG-CONH₂) in the presence of 47 mM CuI, 71 mM L-ascorbic acid, and 20% piperidine. The reaction proceeded for 18 hr at room temperature followed by washing in NMP and copper chelation solution. The N-terminal Fmoc group was removed in 20% piperidine. 110 µmol of 5-hexynoic acid (Sigma), 100 µmol of HATU, and 342 µmol DIEA were added in NMP and the reaction was allowed to proceed at room temperature for 2 hr. After washing with NMP, the 5HA-Biligand-Bio was cleaved from the resin in 95:5:5 TFA:dH₂O:TES and precipitated in diethyl ether. The product was purified by RP-HPLC as a mixture of diastereomers and analyzed by MALDI-TOF mass spectrometry (Expected [M+H]⁺ = 2450.30, Observed [M+H]⁺ = 2449.12)

**Synthesis of Biotinylated Triligand (Figure 3A)**

5HA-Biligand-Bio was assembled and purified as described above. The tertiary ligand (Ac-Az8-RHERI-CONH₂) was synthesized on Rink Amide MBHA resin using as described above. Purification by RP-HPLC gave the desired product (MALDI-TOF: Expected [M+H]⁺ = 961.57, Observed [M+H]⁺ = 961.43). The Triligand was assembled by combining 544 nmol 5HA-Biligand-Bio with 1.09 µmol Ac-Az8-RHERI-CONH₂ in the presence of 600 nmol TBTA, 10 mM CuI, and 30 mM L-ascorbic acid in 4:1 NMP:dH₂O. The reaction proceeded for 18 hr at room temperature with agitation. The desired product was purified by RP-HPLC as a mixture of
diastereomers and analyzed by MALDI-TOF MS (Expected [M+H]^+ = 3410.88, Observed [M+H]^+ = 3408.96).

**Synthesis of Fluorescein-triligand (Figure S16)**

For the synthesis of the fluorescent triligand, 5HA-Biligand-Bio was synthesized as described above. The tertiary peptide Az8-RHERI-CONH₂ was synthesized on Rink Amide resin as described above. Following deprotection of the N-terminal Fmoc group, the resin-bound tertiary peptide was reacted with 1.2 equivalents of 6-[Fluorescein-5(6)-carboxamido]hexanoic acid (Sigma), 1.1 equivalents of HATU, and 3 equivalents of DIEA at room temperature for 30 minutes. Following cleavage from resin, C18 RP-HPLC purification and MALDI-TOF verification of the product, the fluorescein-labeled tertiary peptide was coupled to 5HA-Biligand-Bio via copper catalyzed azide-alkyne cycloaddition by addition of 1 equivalent of fluorescein-labeled tertiary peptide to 1.15 equivalents of 5HA-Biligand-Bio, and 3 equivalents of TBTA in the presence of 10 mM CuI and 30 mM L-ascorbic acid in 4:1 NMP:water. The reaction proceeded for 24 hr at room temperature. The fluorescent triligand was purified by RP-HPLC and analyzed by MALDI-TOF MS: Expected [M+H]^+ = 3841.0, Observed [M+H]^+ = 3840.8.

**Additional Screening Protocols**

**Anchor Ligand**

**Inhibited Target Screen, naïve library:** The naïve library was deprotected, washed in water, and blocked overnight in Akt Blocking Buffer (25 mM Tris-Cl (pH = 7.5), 150 mM NaCl, 10 mM MgCl₂, 0.1% (v/v) β-mercaptoethanol, 0.1% (v/v) Tween-20, and 1 mg/mL BSA. For initial screens with the naïve library, 40 mg of pre-blocked library (~1.14 × 10⁵ sequences) was incubated with Akt-S473-T308P at a final concentration of 21 nM in 1 mL Akt Blocking Buffer. Ac7 was included at a final concentration of 500 µM. The screen proceeded for 75 minutes at room temperature, washed with Akt blocking buffer, and incubated with mouse monoclonal
antibodies specific for phosphorylated T308 ([L32A4], Cell Signaling Technology) for 60 minutes at room temperature. The beads were washed and incubated with alkaline phosphatase-conjugated rabbit anti-mouse antibodies (Promega), and incubated for 60 min. at room temperature. The beads were washed in Akt Blocking Buffer, Akt Wash 1 Buffer (25 mM Tris-Cl, (pH = 7.5), 10 mM MgCl₂, 750 mM NaCl, 0.1% (v/v) Tween-20), and Akt Wash 2 Buffer (25 mM Tris-Cl (pH = 7.5), 10 mM MgCl₂, 150 mM NaCl). The beads were developed in Western Blue Alkaline Phosphatase Substrate (Promega).

**Inhibited Target Screen, focused library:** The initial hit sequences defined a focused library which was similarly screened with 24 mg of focused library in the presence of 500 µM Ac7 and 60 nM Akt-S473E-T308P (3 µg/mL) for 75 min in Akt blocking buffer at room temperature followed by extensive washing in Akt blocking buffer. The L32A4 anti-phospho T308 antibody was added and allowed to bind for 1 hr. at room temperature. After washing, the beads were incubated with anti-rabbit-AP secondary antibody, washed copiously with Akt binding buffer, Akt Wash 1, and Akt Wash 2 buffers, and developed in the presence of BCIP/NBT. The dark purple beads were sequenced as described above.

**Biligand Branch**

**Target Screen, naïve library:** 75 mg of library was incubated with 90 µM biotinylated anchor peptide and either Akt-S473E (9 nM) or Akt-S473E-T308P (37 nM) in 3 mL Akt blocking buffer. The screens were allowed to proceed for 90 min or 24 hr. at room temperature. Screens with Akt-S473E were probed with the 2H10 mAb (CST) while screens with Akt-S473E/T308P were probed with L32A4. Following incubation with alkaline phosphatase-conjugated anti-mouse antibody and development with BCIP/NBT, purple hit beads from the binding screen were stripped overnight, followed by discoloration in DMF. The beads were re-probed with primary
and secondary antibodies and developed in BCIP/NBT as described above. Beads that remained clear were rinsed in dH₂O and stripped overnight.

**Triligand Branch**

**Product Screen, Focused Library:** The focused library was pre-cleared against SA-AP as described previously. Approximately 30 mg. of the pre-cleared library was added to 15 μM 5HA-Biligand-Bio and 21 nM Akt-S473E under blocking conditions. The *in situ* click reaction proceeded for 60 minutes, after which time the beads were washed, stripped with SDS Wash buffer (25 mM Tris-Cl (pH = 7.5), 2% SDS), washed in dH₂O, blocked in Akt blocking buffer, and probed with SA-AP as described above. The beads were developed in the presence of BCIP/NBT and the purple beads sequenced by Edman degradation.

**Determination of In Situ Click yield by Quasi-immuno PCR**

Streptavidin expression was performed according to previously published protocols. Briefly the streptavidin-cysteine (SAC) gene cloned into the pET-3a plasmid was a generous gift from Dr. Takeshi Sano (Harvard Medical School). Transformed BL21(DE3)-pLysE cells were grown at 37°C with shaking in LB medium and selection antibiotics ampicillin and chloramphenicol. The cells were induced at OD600 = 0.6 with IPTG and kept spinning for another 4 hours. The culture was then centrifuged at 1600 x g for 10 min and lysed with lysis buffer (2 mM EDTA, 30 mM Tris-HCl, 0.1% Triton X-100, pH 8.0). The insoluble inclusion bodies were then separated from the lysate by centrifugation at 39,000 x g for 15 min and dissolved in 6 M guanidine-HCl, (pH 1.5) to the original culture volume. The SAC lysate was then refolded by dialysis in 0.2 M Sodium acetate, 10 mM β-mercaptoethanol (β-ME) pH 6.0 overnight before dialyzed against 50mM Sodium bicarbonate, 500 mM NaCl, 10 mM β-ME pH 11 in preparation for column purification. Refolded volumes of SAC were mixed 1:1 with binding buffer (50 mM Sodium bicarbonate, 500 mM NaCl, 10mM β-ME, pH 11). A gravity column packed with 1.5 ml of
iminobiotin agarose resin (Pierce) was washed with 10 ml of binding buffer. The refolded mixture was then applied to the column and the eluted fractions were collected and reapplied to the column again, to maximize SAC recovery. After washing the column with 20 ml binding buffer, SAC was eluted with pH 4 elution buffer (50 mM Sodium acetate, 10mM β-ME). Fractions containing SAC, as monitored by OD$_{280}$, were collected, buffer exchanged to PBS containing 10 mM β-ME, and concentrated to 1 mg/ml final concentration using 10K MWCO filters (Millipore).

Prior to use, stock SAC (streptavidin-cysteine) was buffer exchanged to Tris buffered Saline (TBS) containing 5mM Tris(2-carboxyethyl) phosphine Hydrochloride (TCEP) using desalting columns (Pierce). MHPH (3-N-Maleimido-6-hydrizinumypyridine hydrochloride, Solulink) in DMF was added to SAC at a molar excess of 300:1. In parallel, SFB in DMF (succinimidyl 4-formylbenzoate, Solulink) was added in a 40:1 molar excess to the 5’ aminated oligo (5’…NH$_2$-(CH$_2$)$_6$–GGGACAATTACTATTACAATTACAATGCTCACGTTACGGTCTCGTCTCCCAGG…3’).

The mixtures were allowed to react at room temperature for 3-4 hours. Excess MHPH and SFB were removed and samples were buffer exchanged to citrate buffer (50mM sodium citrate, 150 mM NaCl, pH 6.0) using zeba desalting spin columns (Pierce). The SFB-labeled oligo was then combined in a 20:1 molar excess with the derivatized SAC and allowed to react for 2-3 hours at room temperature before transferring to overnight incubation at 4°C. Unreacted oligos were removed using a Pharmacia Superdex 200 gel filtration column at 0.5 ml/min isocratic flow of PBS. Fractions containing the SA-Oligo conjugates were concentrated using 10K mwco concentration filters (Millipore). The synthesis of SA-Oligo constructs was verified by non-reducing 8% Tris-HCl SDS-PAGE and found to contain 1-2 conjugated oligonucleotides per monomer.
Prior to QPCR, the SA-Oligo was validated in a conventional PCR reaction with biotinylated TentaGel beads. TentaGel beads were synthesized with either a glycine dipeptide (TG-GG) or a glycine dipeptide with an N-terminal biotin (TG-GG-Bio). The beads were blocked in Akt blocking buffer followed by QPCR Blocking Buffer (0.3% (w/v) BSA, 0.1% (v/v) Tween-20, 150 µg/mL sheared salmon sperm DNA (Ambion) in phosphate buffered saline). After 30 minutes the beads were washed and probed with SA-Oligo (0.17 µg/mL) for 60 minutes at room temperature. After washing in QPCR blocking buffer and PBS, single beads were placed in thin-walled PCR tubes. PCR was carried out with Taq polymerase under standard conditions with Forward Primer (5’...TAATACGACTCACTATAGGGACAATTATTTACAATTACA...3’) and Reverse Primer (5’...ACCGCTGCCAGACCCCGATTTGGCCTGGGAGACGAACTCG...3’), both at 100 nM. A small sample was removed every 5 cycles and analyzed for product formation by agarose gel electrophoresis (4% gel). The results are shown in Figure S14.

For the QPCR, 100 nM of each primer (described above) was added to each reaction along with 1X FastStart Universal SYBER Green Master Mix, ROX (Roche). Each cycle consisted of a denaturation step (94 °C for 30 sec), an annealing step (50 °C for 45 sec), and an extension step (72 °C for 60 sec). 30 cycles of PCR were carried out and the Ct value for each reaction determined. A titration series of SA-Oligo was also carried out in the same experiment (duplicate samples) and used to construct a standard curve. A linear fit of the standard curve was used to relate the observed Ct to the amount of SA-Oligo present in the PCR tube.

The following equation (equation 1) was used to obtain the amol SA-Oligo present on each bead from the observed Ct in the QPCR reaction:

\[
(1) \text{amol } SA-\text{Oligo} = \frac{10^{\text{Ct} - 3.372}}{5}
\]

The amount of biligand (amol) of biligand formed on each bead was taken to be the same as the amount of SA-Oligo present.
Inhibition of Akt by Biligand Linker Length Variants

Akt kinase reactions were setup as follows: 0.5 μL of biligand dilution in DMSO or DMSO alone was added to a 20 μL reaction containing 400 ng Akt-S473E-T308P, 200 ng of GST-GSK-3α/β crosstide fusion protein (Cell Signaling Technology), 500 μM ATP, 25 μM Tris-Cl (pH=7.5), 10 mM MgCl₂, 1 mM DTT, 0.01% Triton X-100, 1X Complete protease inhibitors (-EDTA, Roche), 1X PhosStop phosphatase inhibitors (Roche). Reactions proceeded at 30 °C for 30 minutes and were quenched with kinase quenching buffer (500 mM DTT in 20% SDS). 2 μL of each quenched reaction was spotted onto nitrocellulose and the dot blot was blocked with 5% non-fat milk for 1 hr. The blot was probed with rabbit anti-phospho GSK-3α/β (Ser21/9) mAb (37F11, Cell Signaling Technology) at a 1:1000 dilution overnight at 4 °C. The blot was washed and probed with anti-rabbit-HRP secondary antibody at a 1:500 dilution. The blots were developed with Pico West Dura ECL substrate (Thermo) and imaged on film. The image was scanned and each spot was quantitated by densitometry using ImageJ. The total density of was normalized to the density of spots where no inhibitor was added to generate a % pAkt Activity value which was plotted against the log [compound] in GraphPad Prism. In the case of n = 4 and n = 8, the plotted activity was the average of the observed activity for the two diastereomers.

ELISA of Multiligands against immobilized Akt-S473E

3 μg of Akt-S473E was added to each well in a HisSorb Ni-NTA plate (Qiagen) in 50 μL of ELISA blocking buffer (25 mM Tris-Cl (pH = 7.5), 150 mM NaCl, 10 mM MgCl₂, 0.1% (v/v) Tween-20, and 4 mg/mL BSA). 50 μL of imidazole blocking buffer (25 mM Tris-Cl (pH = 7.5), 150 mM NaCl, 10 mM MgCl₂, 0.1% (v/v) Tween-20, 100 mM imidazole, and 4 mg/mL BSA) was added to the control wells. After 18 hr at 4 °C, the wells were washed with ELISA blocking buffer and 50 μL of each ligand dilution was added in ELISA blocking buffer. The ligands were bound at 4 °C for 120 minutes followed by three washes in ELISA blocking buffer. 50 μL of
horseradish peroxidase-conjugated streptavidin (SA-HRP, Thermo) was added (1:5000 dilution in ELISA blocking buffer) and incubated for 70 minutes at 4 °C. The wells were washed three times in TBST (Tris-buffered saline + 0.2% Tween-20) and once in TBS (Tris-buffered saline). 50 µL of peroxidase substrate (KPL) was added to generate the final signal which was quenched in 1 M H₂SO₄ and quantitated on a 96-well plate reader at λ = 450 nm. The Net A₄₅₀ was calculated by subtracting the A₄₅₀ of each blank well (No Akt-S473E) from the experimental well. The data were fit by non-linear regression in GraphPad Prism.

ELISA of Multiligands against His-tagged Kinases

Active, His-tagged enzymes were purchased commercially from the following sources: p70S6 Kinase (R&D Systems), PDK1 (R&D Systems), Abl2 (Sigma), MEK1 (Invitrogen), GSK3β (Invitrogen), and β-Actin (Abcam). Akt1-S473E was expressed and purified as described above. All proteins were diluted in Akt blocking buffer to a final concentration of 24 nM prior to use. Ligands were diluted in Akt blocking buffer to a final concentration of 2.5 µM prior to use. 100 µL of each ligand (250 pmol) was added to each well of a HBC streptavidin-coated 96-well plate. The ligands were bound at 4 °C for 1 hr followed by addition of D-biotin to a final concentration of 500 µM. After 10 minutes at 4 °C, the wells were washed three times with Akt blocking buffer and blocked overnight in 5% non-fat milk.

The wells were washed three times in Akt blocking buffer and 50 µL of active protein was applied to each well. After binding for 120 minutes at 4 °C, the wells were washed three times in Akt blocking buffer to remove unbound protein. 50 µL of horseradish peroxidase-conjugated Anti-His6 antibody was added at a dilution of 1:100 (His Probe (H-3) HRP conjugate, Santa Cruz Biotechnology). The antibody-HRP conjugate was incubated for 60 minutes and the wells were washed 3 times in TBST and once in TBS. 50 µL of peroxidase substrate (KPL) was added and the resulting color change was quenched with 50 µL 1 M H₂SO₄. The A₄₅₀
measured on a 96-well plate reader. The Net A450 was obtained by subtracting the blank value for each protein (No ligand) from each of the triplicate values obtained for the ligand-protein interaction. Each Net A450 value was normalized to the Net A450 from the Akt-S473E samples to obtain a normalized relative binding value. The mean value for the triplicates was calculated and plotted and the error bars were generated from the standard error of the mean (GraphPad Prism).

**Analysis of Triligand Affinity by Surface Plasmon Resonance.**

A Biacore T100 was used for SPR experiments. A Streptavidin Chip (Series S, G.E. Healthcare) was conditioned as recommended by the manufacturer. Biotinylated ligand was diluted into HBSP+ Buffer (G.E. Healthcare) to a final concentration of 100 nM and 137 RU was immobilized on the chip. Akt1-S473E was prepared as described previously and subjected to buffer exchange into HBSP+ using Zeba Desalting Columns (Pierce). Serial dilutions of the enzyme were made in HBSP+ buffer (9000 nM to 1 nM) and flowed over the chip at 50 µL/min. Binding and dissociation were carried out at 10 °C with a contact time of 360 sec, a dissociation time of 400 sec, and a stabilization time of 200 sec. The response was corrected using an unmodified reference flow cell. Kinetic constants were obtained from the sensograms and used to calculate the dissociation constant.

**Determination of Triligand Inhibition Mode and Kinetic Constants.**

Akt-S473E-T308P was prepared by incubating 75 µg Akt-S473E with 1 µg PDK1 (Sigma) in the presence of 500 µM ATP in 1X reaction buffer (25 mM Tris-Cl (pH = 7.5), 10 mM MgCl₂, 2 mM DTT, 1X protease inhibitors (Roche), 1X phosphatase inhibitors (Roche)). The phosphorylation reaction proceeded for 40 minutes at room temperature followed by addition of 25 mM EDTA. The quenched reaction was added to 40 µL Anti-FLAG M2 agarose (Sigma) and allowed to bind for 2 hr at 4 °C. The resin was washed in FLAG Wash Buffer (20 mM HEPES
(pH = 7.4), 150 mM NaCl, 1X protease inhibitors (Roche), 1X phosphatase inhibitors (Roche))
and the Akt1-S473E-T308P eluted with FLAG Elution Buffer (FLAG wash buffer + 0.15 mg/mL
3X FLAG peptide) for 30 minutes at room temperature. The concentration of protein was
determined by Bradford assay.

To determine the inhibition mode of the triligand with respect to the substrate peptide,
kinase reactions were set up with increasing concentrations of peptide substrate (Biotin-
Crosstide, Anaspec). The peptide substrate ranged in concentration from 375 nM to 25 µM (7
concentrations) and the triligand ranged in concentration from 0 – 25 µM (0, 200 nM, 1 µM, 5
µM, 25 µM). The concentration of non-radioactive ATP was held constant at 25 µM and [γ-32P]-
ATP (7000 Ci/ mmol, 10 µCi/µL) was added to a final concentration of 83 nM. Akt-S473E-T308P
was added to a final concentration of 12 ng/µL. Reactions proceeded for 30 minutes at room
temperature in 1X reaction buffer (50 mM Tris-Cl (pH = 7.5), 10 mM MgCl$_2$, 1 mM DTT, 0.01%
Triton-X100, 1X protease inhibitors (Roche), 1X phosphatase inhibitors (Roche)). The reactions
were quenched with Guad-HCl to a final concentration of 3.5M.

Product formation was determined by spotting 5 µL of quenched reaction onto SAM2
Biotin Capture Membrane (Promega), the membrane was washed according to the
manufacturer’s instructions and analyzed by liquid scintillation counting. The observed counts
per minute were converted into pmol of product formed based on the activity and concentration
of the [γ-32P]-ATP assuming a counter efficiency of 50%. The velocity was plotted against
[Peptide] at each [Triligand] and analyzed by linear and nonlinear regression in GraphPad.

To determine the inhibition mode of the triligand with respect to ATP, kinase reactions
were set up with increasing concentrations of ATP. The reactions were set up in a similar
manner to that described above. The concentration of Biotin-Crosstide was held constant at 25
µM and the concentration of enzyme was held at 13 ng/µL. The concentration of ATP ranged
from 125 µM to 4 µM (125 µM, 63 µM, 32 µM, 16 µM, 8 µM, 4 µM) while the concentration of triligand varied from 0 to 10 µM (0, 1 µM, 5 µM, 10 µM). \( [\gamma^{-32}P]\)-ATP (7000 Ci/mmol, 10 µCi/µL) was added to the cold ATP stock to maintain an \([ATP]_{\text{total}}/[\gamma^{-32}P]\)-ATP ratio of 1667. The reactions were incubated, quenched, and analyzed as described above.

**Immunoprecipitation of Akt from OVCAR3 Cell Lysates**

OVCAR3 cells were grown in RPMI-1640 media containing 10% fetal bovine serum, penicillin, and streptomycin. Passage 4 cells were grown to ~ 60% confluence and treated with insulin and EGF at final concentration of 10 µg/mL and 20 ng/mL respectively (induced) or mock treated (control). Cells were grown for an additional 24 hr and then lysed with lysis buffer (10 mM Tris-Cl (pH = 7.5), 100 mM NaCl, 1% (v/v) Triton X-100, 0.1% SDS (w/v), 0.5% deoxycholate, 1 mM DTT, 1 mM EDTA, 1X PhosStop phosphatase inhibitors (Roche), 1X Complete protease inhibitors (Roche). Cell lysate protein concentrations were determined by Bradford assays.

Ligands were immobilized on Streptavidin-agarose by adding 9 µL of 4 mM ligand stock (DMSO) to 50 µL of streptavidin-agarose resin (EMD) pre-blocked in Akt blocking buffer. The mAb resin was prepared by adding 25 µg 5G3 anti-Akt1 antibody (Cell signaling technology) to 100 µL streptavidin resin in Akt blocking buffer. After binding for 1 hr. at 4 °C, 50 µM D-biotin was added to the resin to block any remaining sites.

10 µL of resin-bound ligand was added to a Spin-X filter unit (Sigma) and filtered. To this was added OVCAR3 cell lysate (80 µg protein by Bradford) and Akt blocking buffer to a final volume of 50 µL. Binding occurred at 4 °C for 20 hr with agitation. The resins were washed three times in Akt blocking buffer, three times in Akt Wash 1 buffer, and three times in Akt Wash 2 buffer. The bound material was eluted by adding 40 µL 2X SDS-PAGE loading buffer.
(BioRad) and heating at 94 °C for 10 minutes. A portion of each elution was run on duplicate 12% SDS-PAGE gels (BioRad). One gel was stained with Coomassie (Figure S15) while the other was transferred to nitrocellulose, blocked in 5% non-fat milk, and probed overnight with Rabbit pan-Akt monoclonal antibody (C67E7, Cell Signaling Technology) followed by anti-rabbit-HRP secondary antibody. The primary antibody was used at 1:1000 dilution and the secondary antibody was used at 1:10000 dilution. The blots were developed with Pico West Dura ECL substrate (Thermo) and imaged on film.

Immunofluorescence Microscopy

OVCAR3 cells were grown on poly-lysine coated cover slips, then serum starved for 1 hr. The cells were then treated with either 400 ng/mL EGF (Sigma) and 20 µg/mL insulin (Sigma) or untreated for 10 minutes. Cells were then fixed with 10% formaldehyde for 10 min at 37 °C, washed with PBS, permeabilized by incubating with 0.1% Triton X-100 for 5 min at room temperature, and blocked with 5% goat serum. Permeabilized cells were stained with either a fluorescein-conjugated Pan Akt antibody (R&D Systems IC2055F, 10 µg/mL) overnight or 100 nM fluorescein-triligand for 1 hr. Images were acquired using a Zeiss Pascal 5 Laser Scanning Microscope (Caltech Biological Imaging Center).

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