Supporting Information for:

Identification of a Covalent Binder to the Oncoprotein Gankyrin Using a NIR-Based OBOC Screening Method

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General Materials and Methods

Dimethylformamide (DMF), Dichloromethane (DCM), piperidine, bromoacetic acid (BAA) N,N'-Diisopropylcarbodiimide (DIC), N,N-Diisopropylethylamine (DIPEA), and HBTU, HOBt, and trifluoroacetic acid (TFA), and all amines used for synthesis were purchased from Fisher Scientific. All fmoc protected amino acids were purchased from Novabiochem. Synthesis of all peptoids and the one-bead-one-compound (OBOC) library was performed in fritted syringes purchased from Fisher Scientific. The OBOC library was synthesized on 90 µm TentaGel S NH₂ resin purchased from Rapp Polymere. Peptoids were synthesized on Rink resin LS purchased from Creosalus for validation by an in-gel fluorescence assay. All peptides were purified on an Agilent Technologies 1260 Infinity II reverse phase high performance liquid chromatography (RP-HPLC) system. Purity and identity of each ligand was confirmed with an Agilent Technologies 6120 Single Quadrupole liquid chromatography/mass spectrometry (LC/MS) system. After purification, fractions from the HPLC containing pure ligand were combine and rotovapped. Ligands were then lyophilized overnight and stored at -20 °C, protected from light if conjugated with a fluorophore, until use.

A gankyrin plasmid for bacterial expression was purchased from Addgene and used for expression and purification of the protein (Cat. Number 31332). Plasmid DNA was extracted with a mini-prep kit purchased from Qiagen. DNA was introduced to Rosetta (DE3) Competent cells purchased from Invitrogen. Bacteria was cultured in Luria-Bertani (LB) broth purchased from Fisher Scientific supplemented with ampicillin and chloramphenicol purchased from GoldBio and Fisher Scientific, respectively. Protein was captured with HisPur™ Ni-NTA resin and eluted with imidazole both purchased from Fisher Scientific. Gankyrin was labeled with a NIR-emitting Fluorophore with an IRDye® 800CW NHS Ester Kit purchased from LI-COR (Cat. Number 928-38042). NIR-labeled protein was aliquoted and stored a -20 °C and only thawed immediately before use. Labeled protein was diluted in Intercept (PBS) Blocking Buffer purchased from LI-COR for screening.

Screening was conducted in black 96-well plates with a clear bottom purchased from Fisher Scientific (Cat. Number 265301). After OBOC library beads were individually separated into the wells of the plate, they were blocked with StartBlock™ (PBS) blocking buffer purchased from ThermoFisher Scientific. After incubation, beads were imaged on an Odyssey CLx imaging system purchased from LI-COR. Fluorescence intensities were quantified using ImagesStudio software from LI-COR. Hit peptoids were cleaved from the resin using cyanogen bromide that was purchased from Sigma-Aldrich. Identity of the hit peptoid was determined with a ScieX 4800 MALDI TOF/TOF instrument.

HEK 293T cells were maintained in Dulbecco's Modified Eagle Medium purchased from Corning. Cells were lysed with MPER purchased from Fisher Scientific. Gels from SDS-PAGE for cell culture studies and the in-gel fluorescence studies were imaged on a Sapphire Biomolecular Imager from Azure. Fluorescence intensities were quantified using ImageStudio software from LI-COR.

Synthesizing the OBOC Library

Linker Synthesis

100 mg of 90 µm TentaGel S NH₂ resin was swollen in DMF for 1 hour followed by DCM for 1 hour. The terminal fmoc was removed by adding 2 mL of a 20% piperidine solution in DMF to the resin and allowing it to agitate for 20 minutes at room temperature. A Kaiser test was performed to ensure successful removal of the fmoc and the resin was rinsed three times with DMF followed by three times with DCM. HBTU (4.5 equiv.), HOBt (4.4 equiv.), and Fmoc-methionine-OH (5 equiv.) were premixed in 600 µL of DMF. After dissolving, 10 equiv. of DIPEA was added and the solution was added to the resin. This coupled to the resin for 30 minutes at 37 °C with agitation. The solution was drained, and the resin was rinsed with DMF and DCM. A Kaiser test was performed, producing a negative result to indicate full coupling of the amino acid to the resin. Fmoc was removed by adding 2 mL of a 20% piperidine solution to the resin and allowing it to agitate for 20 minutes at room temperature then the resin was rinsed with DMF followed by DCM. A Kaiser test was performed and produced a positive result, indicating removal of the fmoc. Next, the same equivalences of HBTU, HOBt were weighed followed by Fmoc-Arg(pbf)-OH (5 equiv). These components were dissolved in 600 µL of DMF, then 10 equiv. of DIPEA was added. This solution was added to the resin and allowed to couple for 30 minutes at 37 °C with agitation. The solution was drained, the resin was rinsed with DMF and DCM, and a Kaiser test was performed to ensure successful coupling. The fmoc was removed by adding 2 mL of 20% piperidine in DMF to the resin and agitating for 20 minutes at room temperature. A Kaiser test was performed, and the resin was rinsed with anhydrous DMF. A 2 M BAA and 1 M DIC solution were prepared in anhydrous DMF. Equal amounts were mixed until a precipitate was formed. This was then added to the resin and agitated for 20 minutes at 37 °C. While the resin was activating,
a 1 M stock of bromophenethylamine was prepared by diluting the amine in anhydrous DMF. The BAA/DIC mixture was drained and the resin was washed to remove the precipitate. The 1 M stock of bromophenethylamine was added to the resin and coupled for 1 hour at 37 °C. The solution was then drained and a chloranil test was performed to detect the presence of a primary amine. The same equivalence of HBTU and HOBt as listed above were weighed followed by Fmoc-gly-OH (5 equiv.) and dissolved in 600 µL of DMF. After the solids were dissolved, 10 equiv. of DIPEA was added to the solution and this was added to the resin. The amino acid residue coupled for 30 minutes at 37 °C. The solution was drained, the resin was rinsed with DMF and DCM, and a Kaiser test was performed to ensure the residue had successfully coupled. Fmoc was removed by adding 2 mL of a 20% piperdine solution in DMF to the resin and letting it agitate for 20 minutes at room temperature. A Kaiser test was then performed to ensure the fmoc removal was complete. The same equivalence of HBTU and HOBt as listed above were weighed followed by 5 equiv. of N-[(9H-Fluoren-9-ylmethoxy)-carbonyl]-L-propargylglycine. This was dissolved in 600 µL of DMF followed by 10 equiv. of DIPEA. The solution was added to the resin and allowed to couple for 30 minutes at 37 °C with agitation. The solution was drained and the resin was rinsed with DMF followed by DCM and a Kaiser test was performed.

A small clump of resin was transferred to a 1.5 mL eppitube and the synthesized linker was cleaved from the resin by adding 1 mL of a 95% TFA, 2.5% DCM, and 2.5% Triisopropylsilane (TIPS) solution. This agitated for 1 hour at room temperature. The TFA was blown off with argon gas, then the cleaved product was resuspended in a 50/50 mixture of ultrapure water and acetonitrile (ACN) substituted with 0.1% formic acid (FA). The dissolved product was run on LC/MS to ensure the linker structure had been properly synthesized. The resin was stored at 4 °C until use with the terminal fmoc still attached. Right before the library was synthesized, the fmoc was removed by adding 2 mL of a 20% piperdine solution to the resin and allowing it to agitate for 20 minutes at room temperature. The resin was then rinsed with DMF followed by DCM and a Kaiser test was performed to ensure successful deprotection.

**OBOC Library Synthesis**

The 100 mg of linker resin synthesized as described above was swollen in DMF for 30 minutes followed by DCM for 30 minutes. The terminal fmoc was removed as described above. The resin was washed once with anhydrous DMF then a 2 M stock of BAA and a 1 M stock of DIC in anhydrous DMF was prepared. Equal amounts of the solutions were mixed until a precipitate formed and this was added to the resin. The resin was activated for 15 minutes at 37 °C with agitation. During this time, 0.5 M stocks of the seven amines listed in manuscript at position 1 were prepared by diluting the amines in anhydrous DMF. The activating solution was drained from the resin and the resin was rinsed with anhydrous DMF then split roughly equally between seven syringes. One of the diluted amines was added to each syringe and coupled for 1 hour at 37 °C with agitation. The amine solutions were then drained, and the resin was rinsed three times with anhydrous DMF. A chloranil test was performed for each syringe to ensure successful coupling of each amine. The resin was then pooled back into one syringe and activated using the premixed 2 M BAA and 1 M DIC solution as described above. Resin was then roughly equally separated into seven syringes and 0.5 M stocks of the amines listed for position 2 in the manuscript body were added. Amines coupled for 1 hour at 37 °C with agitation. The solutions were then drained, and the resin was washed 3 times with anhydrous DMF. A chloranil test was performed for each syringe, then the resin was pooled back together. The resin was activated with the 2 M BAA and 1 M DIC solutions as described above. After activation, however, the resin was not split into separate syringes, as the same amine was going to be coupled for all the structures in the 3rd position. 4-(2-aminoethyl)benzensulfonyl fluoride hydrochloride was weighed such that dissolving in 500 µL of anhydrous DMF would produce a 0.5 M stock. After dissolving the amine, DIPEA was added to a final concentration of 0.5 M to quench the hydrochloride. This was then added to the resin and allowed to couple for 1 hour at 37 °C with agitation. The amine was then removed, resin was washed three times with anhydrous DMF and a chloranil test was performed. The resin was then activated with the 2 M BAA and 1 M DIC as described above. The resin was then roughly equally split between 7 syringes and 0.5 M stocks of amines listed at position four in the manuscript body were added to one of the syringes. This incubated for 1 hour at 37 °C with agitation. The solution was drained, the resin was washed three times with anhydrous DMF, and a chloranil test was performed for each syringe. Resin was then combined into one syringe and the pbf protecting group on the arginine in the linker was removed by adding 1 mL of a 95% TFA, 2.5% TIPS, and 2.5% DCM solution. This agitated for 1 hour at room temperature. The solution was drained and the resin was washed 3 times with DMF followed by 3 times with DCM. The resin was stored at 4 °C until use.
A His-tagged gankyrin plasmid resistant to ampicillin was purchased from AddGene. An 8 mL starter culture was prepared by scraping bacteria from the agar stab and inoculating it in LB broth with 0.1 mg/mL of ampicillin. The culture grew overnight at 37 °C. The following morning the plasmid DNA was extracted with a mini-prep kit following the manufacturer's protocol. Rosetta (DE3) competent cells were transformed with the plasmid DNA following the manufacturer's protocol. Competent bacteria were streaked on a LB agar plate supplemented with ampicillin and allowed to grow overnight at 37 °C. The next day, single bacterial colonies were inoculated into 8 mL of LB broth supplemented with ampicillin and chloramphenicol and allowed to grow overnight at 37 °C. Glycerol stocks of the transformed bacteria were prepared by diluting 100 µL of the bacteria stock in 100 µL of a sterile 50% glycerol solution. Glycerol stocks were stored at -80 °C.

To purify gankyrin, 20 µL of the glycerol stock was inoculated into 8 mL of LB broth supplemented with ampicillin and chloramphenicol and grew overnight at 37 °C with agitation. 1 L of LB broth was sterilized by autoclaving and once the media had cooled, 100 mg of ampicillin was added to the broth. The entire 8 mL starter culture was then added to the broth and agitated at 37 °C. Bacteria grew until and OD_600 of 0.6-0.8 was reached. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was dissolved in phosphate-buffered saline (PBS) such that the final concentration in the 1 L flask would be 1 mM. Protein production was induced with IPTG for 2.5 hours at 37 °C. Then, bacteria were pelleted by centrifuging at 4,700 xg for 15 minutes. Media was poured off and bacteria were resuspended in 40 mL of lysis buffer (50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 0.1% triton-X 100, 1 mM EDTA supplemented with 0.25 mg/mL lysozyme and 1 mM phenylmethylsulfonyl fluoride (PMSF)). Bacteria was transferred to a 50 mL falcon tube and ultrasonicated at 30% amplitude for 1 minute with 3 seconds on and 2 seconds off. Ultrasonication was carried out at 4 °C and the falcon tube was placed in ice. Lysate was clarified by pelleting at 14,000 xg for 40 minutes at 4 °C in a prechilled centrifuge. Supernatant was collected in a new tube. Gankyrin was purified by allowing the lysate to incubate with 200 µL of HisPur™ Ni-NTA resin for 40 minutes at room temperature with gentle agitation. Lysate was then drained and the resin was washed three times with 10 mL of PBS to remove protein that was bound nonspecifically. 10 mM, 50 mM, 150 mM, 350 mM, and 600 mM solutions of imidazole were prepared by diluting imidazole in PBS. Two 1 mL imidazole fractions of each concentration were added to the resin and collected in 1.5 mL eppitubes starting with the 10 mM concentration. 42 µL from each fraction was mixed with 8 µL of 4X Laemml buffer and samples were heated for 5 minutes at 95 °C. Samples were run on SDS-PAGE and the gel was stained with Coomassie (Supporting Information Figure S1A). Fractions containing only pure gankyrin (protein near 24 kDa) were compiled and added to a dialysis bag. The pure protein dialyzed in PBS overnight at 4 °C. A western blot was conducted using an anti-HIS antibody (Novus Biologicals) the first time the protein was subjected to western blot analysis using an antibody that recognizes the HIS tag. (B) Fractions containing pure gankyrin were combined and then subjected to western blot analysis using an antibody that recognizes the HIS tag.

1 mg of gankyrin was labeled with the NIR fluorophore using an IRDye® 800CW NHS Ester Kit purchased from LI-COR following the manufacturer's protocol. Briefly, the pH of the protein solution was raised to 8.5 by adding 1 M potassium phosphate buffer, pH 9.0. This ensured the NHS ester moiety on the dye only bound the N-terminus of the protein. Next, a vial of 800CW dye was dissolved in 25 µL of ultrapure water. The correct amount of dye was calculated using the manufacturer's protocol and was added to the gankyrin solution. The dye incubated with the gankyrin for 2 hours at 4 °C. Excess dye was removed by passing the solution through a desalting column. The absorbance at 780 and 280 nm of the solution was measured with a NanoDrop One system and used to calculate the dye to protein ratio using the manufacturer's protocol. The dye to protein ratio...
was determined to be 0.7. The protein was aliquoted and stored at -20 °C. Protein was thawed and used immediately before use.

**Screening of Gankyrin Against the OBOC**

Beads of the OBOC were stored at 4 °C until use. Prior to screening beads were conditioned to PBS. Beads were first swollen in DMF for 20 minutes. The solution was drained and replaced with a 25% DMF, 75% PBS solution for 30 minutes. Beads were agitated until clumps no longer formed prior to increasing the amount of PBS. If the beads did clump, the amount of PBS was reduced, and beads were agitated until clumps no longer formed. Beads were stored dry at 4 °C after the initial transfer to PBS. Beads were allowed to swell in 100% PBS for 20 minutes after each removal from the refrigerator to ensure they would not collapse during screening.

Beads were individually split into the wells of a black 96-well plate with a clear bottom by adding 1 mL of PBS to the syringe with the library and cutting the tip off of a 200 µL micropipette tip and looking at the well plate under a microscope. 50 µL of PBS was added to each well of the plate. About 20 µL of the library in PBS was pipetted with the cut micropipette tip and added to one well of the plate. Then, about 4 µL of beads from the first well were transferred to the second well. Individual beads in the second well were then transferred to the wells of the plate. PBS was then removed from the wells by careful pipetting. 50 µL of blocking buffer purchased from ThermoFisher (see general methods for full name) was added to each well. Beads blocked for 30 minutes at room temperature with gentle agitation. The blocking buffer was then removed by pipetting.

An aliquot of NIR-labeled gankyrin was removed from the freezer immediately before use and diluted in Intercept Blocking buffer (PBS) from LI-COR. 50 µL of the protein diluted in blocking buffer was added to each well. The final amount of labeled gankyrin per well was 0.6 ng. The plate was covered with a lid and wrapped in tin foil. The beads incubated with the labeled protein overnight at 4 °C with gentle agitation. The following day the protein solution was removed and wells were washed with 50 µL of PBS. Beads were then resuspended in 50 µL of PBS and imaged on an Odyssey CLx imaging system from LI-COR. Importantly, the resolution of the scan was set to 84 µm to accommodate visualizing the 90 µm beads. Fluorescence intensities of individual beads that resulted from the labeled protein binding the small molecule were quantified using ImageStudio software from LI-COR. Fluorescence intensities were normalized to the intensity of a well containing no bead to accommodate for background fluorescence. Two screens of 424 beads were performed. The top 5% fluorescence intensities from each screen were considered initial hits. Hits were then identified as follows.

**Hit Identification from Initial Screen**

After selecting the top 5% of fluorescence intensities as hits, beads in the corresponding wells were removed from the screening plate and placed into a chemical resistant plate by pipetting. Since the binding between the protein and small molecule is covalent, the protein was first removed by agitating beads in increasingly organic solvent to unfold the protein and promote disassociation with bead. First, beads were washed three times with 50 µL of ultrapure water. Beads were agitated for 10 minutes during each wash. Next, beads were washed three times for 10 minutes in a solution of 50/50 ultrapure water and DMF. Next, beads were washed three times for 10 minutes with DMF. Finally, the beads were agitated overnight with 50 µL of DCM at room temperature. The plate was tightly wrapped with tin foil to prevent evaporation. The next day, the DCM was removed. The small molecule on the bead was cleaved with a 50 mg/mL solution of cyanogen bromide dissolved in a 5:4:1 ACN: Glacial Acetic Acid (GAA):Water. 50 µL of this cocktail was added to each well. The plate was wrapped in tin foil to prevent evaporation and agitated overnight in a chemical hood. The following morning, the plate was speed vacced to dry the cleaved product. The product was re-dissolved in 7 µL of a 50/50 ACN/Ultrapure water solution with 0.1% TFA. 1.3 µL of this was spotted with 0.7 µL of MALDI matrix solution. Hits were subjected to MALDI-TOF analysis. The unique isotope provided by the bromine in the linker structure facilitated identification of amines from the hit structures from other fragments associated with left over protein or other sample contaminants. Several hit structures were identified from each independent screen. The structures between the two screens were combined and peptoids that exhibited similarity in the amines at various positions were further pursued for validation. This produce 10 structures between the two screens that were further validated for binding to gankyrin with an in-gel fluorescence assay. Sample structures from both screens and the resulting overlapping structures from combining both screens can be seen in Supporting Information Appendix S1.
Synthesis of Ligands for In-Gel Fluorescence Validation

After identification of the hit structures by MALDI-TOF, they were resynthesized on a linker structure conjugated to fluorescein. These ligands were used for an in-gel fluorescence assay in which purified gankyrin bound fluorescently labeled ligand.

Linker Synthesis

1 g Rink amide resin purchased from Creosalus was swollen in DMF for 30 minutes followed by DCM for 30 minutes. The fomoc was removed from the resin by adding 5 mL of a 20% piperidine solution in DMF and agitating the resin for 20 minutes at room temperature. A Kaiser test was performed to ensure successful deprotection. HBTU was equilibrated to room temperature then 4.5 equivalence was weighed. 5 equivalence of DIPEA was added to the solution and this was added to the resin. This was allowed to couple for 1 hour at room temperature then an additional hour at 37 °C. The solution was drained, and resin was washed three times with DCM followed by three times with DMF. A Kaiser test was performed to ensure successful coupling. The fomoc was removed from the cysteine by adding 5 mL of a 20% piperidine solution in DMF and allowing it to agitate with the resin for 20 minutes at room temperature. A Kaiser test was performed to ensure full deprotection of the resin. Next, 4.5 equivalence of HBTU was weighed followed by 5 equivalence of N-[9H-Fluoren-9-ylmethoxy]-carbonyl]-L-propargylglycine (minipeg). This was dissolved in 10 mL of DMF and 10 equivalence of DIPEA was added. The solution was added to the resin and coupled for 2 hours at 37 °C. The solution was then drained and the resin was washed three times with DMF followed by three times with DCM. A Kaiser test was performed to ensure successful coupling. The fomoc was removed from the linker by adding 5 mL of a 20% piperidine solution in DMF and allowing it to agitate with the resin for 20 minutes at room temperature. A Kaiser test was performed to ensure full deprotection of the resin. The activating solution was removed and the resin was washed with DCM for 2 hours at 37 °C. This resin was then transferred to an epipette and the linker was cleaved from the resin with a 95% TFA, 2.5% TIPS, 2.5% DCM solution. This agitated for 1 hour at room temperature. TFA was blown off with argon and the cleaved product was resuspended in 200 µL of a 50/50 ACN/ultrapure water solution with 0.1% FA. This was injected on an Agilent Single quadrupole LC/MS to check the mass. Resin was then roughly equally separated in syringes such that the final amount per syringe was about 50 mg. Resin was stored at 4 °C.

Synthesizing Hit Structures on the Cys(MMT) Linker

50 mg of resin containing the linker described above was swollen in DMF for 30 minutes followed by DCM for 30 minutes. The fomoc was removed from the mini-peg by adding 1 mL of a 20% piperidine solution in DMF and agitating it for 20 minutes. A Kaiser test was performed to ensure successful deprotection. The resin was washed with anhydrous DMF. Next, a 2 M BAA and 1 M DIC solution was prepared and equal volumes were mixed until a white precipitate formed and the solution was added to the resin. This incubated for 15 minutes at 37 °C. 500 µL of a 0.5 M solution of the first amine of each structure was prepared by diluting it in anhydrous DMF. The activating solution was removed and the resin was rinsed once with anhydrous DMF and the amine solution was added to the syringe. The amine coupled for 1 hour at 37 °C. The resin was then washed with anhydrous DMF and a chloranil test was performed to ensure successful coupling. This procedure was repeated for all four positions. For the 4-(2-aminoethyl)benzensulfonyl fluoride hydrochloride, 1.5 equivalence of the solid was weighed and dissolved in 250 µL of anhydrous DMF. DIPEA was added to a

Supporting Information Figure S2: Example Images of Gels From In-Gel Fluorescence Validation Assay. (A) Examples of gels obtained during the in-gel fluorescence validation experiment. The fluorescence intensities of the bands shown in the gels on the left were quantified to produce the graphs on right. (B) Quantification of the gels provided in Figure 4 of the manuscript body. Since TMM-6 appeared to produce higher fluorescence intensities at some of the concentrations compared to the negative control, this was repeated in experimental triplicate.
final concentration of 0.5 M. This was added then added to the syringe and coupled for 1 hour at 37 °C.

After all the amines were coupled, the MMT protecting group was removed from the cysteine on the linker to conjugate fluorescein. 1 mL of a 2% TFA solution in DCM was added to the syringe and agitated or 5 minutes. The solution was drained and replaced until it no longer turned yellow. Then, the pH of the resin was neutralized by washing with a 10% DIPEA solution in DCM three times for 5 minutes. 1.5 equivalence of N-(5-Fluoresceinyl)-maleimide was weighed and dissolved in 250 µL of DMF. This was then added to the syringe and coupled for 1 hour. The solution was drained and the resin was washed 3 times with DMF followed by 3 washes with DCM. The resin was stored at 4 °C until purification. The peptoid was cleaved from the resin by adding a 95% TFA, 2.5% TIPS, 2.5% DCM solution to the resin and agitating it for 2 hours at room temperature. The cleaved produce was collected in a clean 15 mL Falcon tube and the resin was rinse twice with 1 mL of DCM. The TFA was blown off with argon. The cleaved product was resuspended in 200 µL of a 50/50 ACN/ultrapure water solution with 0.1% TFA. The product was then purified by RP-HPLC and the purity was checked by LC/MS (Supporting Information Appendix S2). After purification, HPLC fractions containing the pure product were combine and rotopapped to remove residual ACN. The product was then frozen and lyophilized. The resulting powder was stored at -20 °C protected from light until use.

In-Gel Fluorescence Validation

Gankyrin was purified as described above. Immediately prior to the start of the experiment, fluorescently labeled negative control or the test ligand were removed from the freezer and a small amount of powder was scrapped from the vial and placed in a 0.6 mL tube. Ligands were dissolved in 6 µL of DMSO then diluted 1:100 in PBS. The concentration of the ligands were determined by measuring absorption at 494 nm and Beer's Law. Stocks of each ligand were prepared such that the final concentrations in the samples would be 20, 8, 1, or 0.1 µM by diluting the ligands in DMSO. Purified gankyrin was concentrated to a 10.2 ng/µL stock. 49 µL of the protein was added to 0.6 mL eppitubes. Next, 1 µL of the negative control or test ligands at each concentration were added to one sample of protein. As a control, one sample was also dosed with 1 µL of DMSO. The final protein amount was 500 ng per sample. Samples were vortexed to mix then incubated at 4 °C for three hours with gentle rotation, protected from light. During incubation, 12.5% resolving SDS-PAGE gels were prepared with a 4.5% stacking gel. The reaction between the ligands and the protein was quenched by adding 20 µL of 4X Laemmli buffer and heating at 95 °C for about five minutes. Samples were then run on SDS-PAGE in chambers protected from light. The gel was run at 120 V for about 1.5 hours to prevent streaking of the excess ligand in the gel. Gels were run until all Laemmli dye was run off the bottom. The gels were then washed three times with ultrapure water.

Gels were imaged on an Azure Sapphire Biomolecular Imager (Supporting Information Figure S2A). The intensity of the fluorescing bands resulting from the labeled ligands binding the purified gankyrin were quantified using ImageStudio software from LI-COR. The intensities of the negative control band and test ligands were compared. Quantification of the fluorescence intensities of the bands produced by TMM-1 and TMM-6 shown in the manuscript body are provided as well (Supporting Information Figure S2B). This experiment was repeated in experimental triplicate with TMM-6 and the intensities for each concentration were normalized by dividing signals by the signal of the DMSO control and the data was graphed (Supporting Information Figure S3).

Competition of Fluorescent and Non-Fluorescent TMM-6

Supporting Information Figure S3: Replicates of TMM-6 In-Gel Fluorescence Assay. Since TMM-6 produced higher fluorescence intensities than the negative control during the initial validation, the experiment was conducted in triplicate. Gels from each of the three replicates are shown above. The fluorescence intensities of each band were quantified and graphed as shown on the right. In Figure 4C of the manuscript body, the values were obtained by dividing each of the signals by the respective DMSO control. This was then combine to produce a triplicate value for each concentration of TMM-6 and the negative control and graphed.
To ensure the linker structure or fluorescein was not what was responsible for binding of TMM-6 to gankyrin, we conducted a competition experiment in which either fluorescently labeled, or unlabeled TMM-6 incubated with purified gankyrin, then the other version of the ligand was added. Fluorescently labeled TMM-6 was synthesized exactly as described above. Unlabeled TMM-6 was synthesized on rink amide resin exactly as described above, but the peptide was conjugated directly to the resin with no linker structure. Both versions of the ligand were purified by RP-HPLC and purity was checked by injection on an Agilent single quadrupole LC/MS system. Purified ligands were stored -20 °C until use. Unlabeled TMM-6 was then dissolved in DMSO to make a 5 mM stock which was stored at the same temperature until use. Gankyrin was purified the day before use as described above.

On the day of the experiment, an aliquot of unlabeled TMM-6 was thawed. A small amount of lyophilized fluorescently labeled TMM-6 was scrapped from the vial and dissolved in 5 µL of DMSO. This was diluted 1:100 in PBS and the concentration was determined by measure absorbance at 494 nm using Beer’s Law. Labeled and unlabeled ligand was diluted in DMSO to make two master stocks, one at 1 mM and one at 0.5 mM. Gankyrin was concentrated such that the final amount in each sample would be 500 ng. 49 µL of purified protein was added to 8 0.6 mL tubes. Next, 0.5 µL of the 1 mM fluorescently labeled TMM-6 was added to three tubes of protein. 0.5 µL of the 1 mM unlabeled TMM-6 was added to 3 protein samples. As a positive control, 0.5 µL of the 1 mM fluorescently labeled TMM-6 was added to a tube. As a negative control, 1 µL of DMSO was added to a protein sample. Samples were vortexed to mix then incubated at 4 °C for one hour. After this first incubation, 0.5 µL of the 0.5 mM stock of TMM-6 was added to the samples already containing the fluorescent ligand. Likewise, 0.5 µL of the 0.5 mM fluorescently labeled TMM-6 was added to the samples already containing the unlabeled ligand. The samples were then returned to 4 °C and incubated for an additional hour.

SDS-PAGE gels were made as described above. 20 µL of 4X Laemmli buffer was added to each sample to quench the reaction and samples were heated at 95 °C for five minutes. 50 µL was loaded into the wells of the gel and the gel was run at 120 V for about 90 minutes, until all dye had run off the bottom. Gels were then washed 3 times with ultrapure water and imaged with a Sapphire Biomolecular Imager from Azure. Fluorescence intensities that resulted from the fluorescently labeled ligand binding the gankyrin were quantified with ImageStudio software from LI-COR. We expected to find that fluorescence of samples dosed first with the unlabeled ligand, followed by the fluorescently labeled ligand would be significantly lower than the adding the fluorescent ligand first, followed by the unlabeled ligand. This would indicate that both ligands were binding in the same area on gankyrin and binding was not due to nonspecific interactions between the protein and the fluorescein on the labeled ligand. Indeed, we found significantly lower fluorescence intensities when the protein was first dosed with the unlabeled ligand, confirming the linker structure or fluorescein were not responsible for binding (Supporting Information Figure S4).

**Binding of Covalent and Non-Covalent Tmm-6 Monitored by In-Gel Fluorescence**

To ensure binding of the ligand to gankyrin was able to withstand SDS-PAGE analysis because of the covalent amine and not other nonspecific interactions between the peptoid and protein, a version of TMM-6 was synthesized with the fluorescein linker that exchanged the 4-(2-aminoethyl)benzensulfonyl fluoride for 2-phenylethylamine. This amine is very similar in structure but does not contain the sulfonyl fluoride moiety that facilitates covalent binding of the ligand to the protein. Both the covalent and noncovalent ligand were
synthesized exactly as described above and were purified by RP-HPLC. Samples were injected into an Agilent single quadrupole LC/MS system to check purity. Pure lyophilized product was stored at 4 °C protected from light until use. Gankyrin was purified as described above one day prior to the experiment.

On the day of the experiment, a small amount of the covalent and noncovalent ligand were scrapped from the vial and dissolved in DMSO. Samples were diluted 1:100 in PBS and the concentration of each stock was determined by measuring absorbance at 494 nm and Beer’s Law. Master stock solutions of each ligand were created such that the final concentrations in the samples would be 20, 8, 1, and 0.1 µM. 49 µL of purified protein was added to 0.6 mL tubes. The samples would be 20, 8, 1, and 0.1 µM. 49 µL ofLaw. Master stock solutions of each ligand were by measuring absorbance at 494 nm and Beer’s Law. 400 µM stocks of each ligand were scrapped from the vial and dissolved 6 µL of DMSO. This was diluted 1:100 in PBS and the concentration of the sample was 500 ng. Lysate was diluted in PBS and 46.3 µL of lysate was added to 0.6 mL tubes. The final lysate amount in each sample was 50,000 ng. Next, 2.7 µL of gankyrin was added to each tube, making the final amount of gankyrin in each sample 500 ng, or 1% of the total protein concentration. Fluorescently labeled TMM-6 and negative control was incubated pure gankyrin with TMM-6 or a version of TMM-6 without the covalent amine. Samples were then subjected to SDS-PAGE and the gels were imaged for fluorescence as shown on the left side. The fluorescence intensity of each band was quantified and divided by the signal from the control lane consisting of gankyrin incubated with DMSO. The values for each concentration in each replicate were then combined and graphed as shown on the right. As expected, significantly higher fluorescence intensities were produced by incubating gankyrin with TMM-6 as compared to the noncovalent version of TMM-6. This indicated that the sulfonyl fluoride moiety produced the covalent interaction necessary to visualize gankyrin bound to the fluorescent ligand.

Supporting Information Figure S5: Binding of Covalent and Non-Covalent TMM-6 to Gankyrin. To ensure the sulfonyl fluoride was facilitating covalent binding that allowed us to observe binding of the ligand to gankyrin by SDS-PAGE, we incubated pure gankyrin with TMM-6 or a version of TMM-6 without the covalent amine. Samples were then subjected to SDS-PAGE and the gels were imaged for fluorescence as shown on the left side. The fluorescence intensity of each band was quantified and divided by the signal from the control lane consisting of gankyrin incubated with DMSO. The values for each concentration in each replicate were then combined and graphed as shown on the right. As expected, significantly higher fluorescence intensities were produced by incubating gankyrin with TMM-6 as compared to the noncovalent version of TMM-6. This indicated that the sulfonyl fluoride moiety produced the covalent interaction necessary to visualize gankyrin bound to the fluorescent ligand.

Pulldown of Gankyrin in Spiked HEK 293T Cell Lysate

To determine if TMM-6 could recognize and bind gankyrin in more complex protein mixtures and evaluate the specificity of TMM-6, we conducted a binding experiment in which fluorescently labeled ligand incubated with HEK 293T cell lysate spiked with purified gankyrin. Fluorescently labeled TMM-6 and the negative control were synthesized as described above and purified by RP-HPLC. After checking purity by LC/MS, the lyophilized product was stored at -20 °C protected from light with gentle rotation. After incubation, the reaction was quenched by adding 20 µL of 4X Laemmli buffer and

**Supporting Information S5**
heating samples at 95 °C for 5 minutes. 50 µL of each sample was subjected to SDS-PAGE using a freshly made gel as described above. The gel was run at 120 V for about 90 minutes, until the loading buffer ran off the bottom. The gel was then washed 3 times with ultrapure water and imaged on a Sapphire Biomolecular Imager from Azure (shown in the manuscript body). Two bands produced the highest fluorescence intensities after incubating with TMM-6. These were excised from the gel by cutting with a clean razor blade and placed in clean 1.5 mL eppitubes. The gel was then rescanned to ensure the entire band had been successfully cut from the gel. The gel fragments were submitted to the Purdue Proteomics Facility and the most abundant protein in each band was determined to be gankyrin. Since purified gankyrin was added to the samples, it is unclear why it produced two bands differing in molecular weights in the spiked HEK 293T lysate samples. It is possible that one band represents gankyrin that has been modified by other enzymes in the lysate, resulting in a different molecular weight. Notably, there were other fluorescent bands in the TMM-6 sample, indicating that this ligand may bind other proteins. However, none of the bands fluoresced to the same extent as the bands identified as gankyrin, indicating that this interaction is the most prominent in the lysate. Binding of the negative control to protein in the spiked lysate produced only a few bands, indicating that is does not bind many proteins to a significant extent.

Appendix S1: Identification of Hit Structures from Gankyrin Screens

The following data represents several examples of hit structures identified from the first screen, second screen, and structures that overlapped between both screens. Overlapping refers to amines that were present in the same position between both screens. The overlapping structures were further pursued for the in-gel validation. The data was obtained by MALDI-TOF as described in the Hit Identification from Initial Screen section.
Screen 2

Exact Mass: 1302.59

Screen 2

Exact Mass: 1358.54
Overlapping Structure from Screen 1 and 2, **TMM-6**

Exact Mass: 1250.53

Overlapping Structure from Screen 1 and 2

Exact Mass: 1326.53
Appendix S2: Example Traces LC/MS Trace of Fluorescently Labeled Negative Control and Ligands

The following LC/MS traces are example structures of the fluorescently labeled ligands that were used to conduct the in-gel validation assay. The structures tested were the overlapping structures from both screens against gankyrin. The ligands were synthesized following the protocol described in Synthesis of Ligands for In-Gel Fluorescence Validation section. The data was obtained by injecting RP-HPLC fractions on an Agilent single quadrupole LC/MS system.

TMM-1
Negative Control