Supporting Online Material

Supporting Online Material: Materials and Methods

Light Scattering. Fresh Aβ(1-42) (Anaspec, San Jose, CA) was prepared by resuspending the peptide in 100 mM NaOH pH 9.5 to 10, sonicating for 5 minutes, and passing the resulting solutions through a 10 kDa filter (YM-10; Millipore, Bedford, MA) (*S1*). Stock solutions (100 μM in 2 mM NaOH) were stored at –80 °C and diluted to 25 μM with PBS pH 7.2 to initiate fibrillogenesis. Identical methods were used to prepare the Aβ(1-42) solutions utilized for TEM, AFM, MTT, Tunel and thioflavin T experiments. Turbidity was measured at 30 °C with orbital shaking in half area, non-binding surface 96-well plates (Corning) on a TECAN GENios plate reader. The total volume was 50 μL and the concentration of FKBP was 1 μM. The results are representative of two independent experiments each performed in triplicate.

Thioflavin T. Compounds (CR or SLF-CR), FKBP (1 μM), and Aβ (25 μM) were incubated in a total volume of 10 μL PBS pH 7.2 in black Corning 96-well plates. After a 96 hour incubation in the dark at 22 °C, thioflavin T (200 μL of 5 μM thioflavin T in 50 mM glycine pH 8.5) was added. Fluorescence was measured on a SpectraMax Gemini (Molecular Devices, Sunnyvale, CA), using an excitation wavelength of 446 nm (± 5 nm) and an emission of 490 nm (± 5 nm). The data was fit to sigmoidal curves using DeltaGraph (DeltaPoint, Inc) and the IC_{50} for each treatment is shown. The percent aggregation is arbitrarily defined at the bottom (0%) by the fluorescence of a solution of thioflavin T to which no Aβ has been added (Em_{490nm} = 3.3) and at the top (100%) by the fluorescence of a solution containing Aβ but no inhibitors (Em_{490nm} = 42).

A linear relationship between thioflavin T fluorescence and percent aggregation is used for simplicity.

Transmission Electron Microscopy. TEM was conducted using 300 mesh formvar-coated copper grids and 1% uranyl acetate stain on a JEOL TEM1230 and imaged with a Gatan 967 slow-scan CCD at 80 kV. Samples were incubated for 4 days at 22 °C in PBS pH 7.2 containing 25 μM Aβ(1-42), 1 μM FKBP and 1 μM drug, where appropriate. Compounds from the linker series were used at 0.5 μM.

Atomic Force Microscopy. Samples for AFM were prepared as for TEM, above, applied to a clean silicon surface, washed twice with dH_2O, dried, and imaged at a scan rate of 1 Hz using a silicon probe (TESP) on an AFM Nanoscope Dimension 3000 (Digital Instruments) in Tapping Mode. The dimensions of the globular aggregates in the samples treated with SLF-CR/FKBP were approximated using NIH Image.

Cell Viability Assay. Rat hippocampal (E19.5 and P0) neurons were prepared as previously described (*S2*). Fresh Aβ(1-42) (100 μM) was prepared under basic conditions (see Fig. 1) and incubated with inhibitors for two days at 22 °C in PBS pH 7.2 and then diluted 1:4 with culture media and applied to neurons. MTT assays were conducted as per manufacturer's specifications after two additional days of incubation at 37 °C and 5% CO_2. The inhibitor concentrations are the in vitro incubation values.

Immunofluorescence Microscopy. Rat hippocampal neurons (P0) were cultured 10 days on Matrigel-coated glass coverslips (Deckgläser, Germany) in 24-well plates (Corning) at 37 °C and 5% CO_2. Aβ solutions were prepared and applied as above. The final concentration of Aβ was 25 μM and drug and FKBP were present at 1 μM. Following a two-day incubation, cells were prepared for microscopy as described (*S2*).

Surface Plasmon Resonance: Amide coupling was used to generate a carboxymethyldextran (CM5; BIAcore, Uppsala, Sweden) sensor chip bearing a mock-immobilized surface on flow chamber 1 (Fc1), 950 RU of Aβ(1-40) (AnaSpec, San Jose, CA) on Fc2, and 2800 RU of bacterially-expressed recombinant FKBP12 on Fc3. The regeneration conditions were as follows: for removing analyte from Aβ surfaces (4 M guanadine HCl, 10 mM Tris buffer pH 8.0) and for removing analyte from the FKBP surface (750 mM NaCl, 250 mM NaOH). To calculate K_{D1} and K_{D2} (± SEM) for Aβ binding (in part B), the control signal was subtracted and data was fit according to the method of Cairo *et al.* (see Fig. S1) (*S3*). In each case, eight concentrations of analyte distributed around the K_D were injected in duplicate in the running buffer (HBS-EP; BIAcore). Congo red is not uniquely specific (*S4*) for Aβ and, consistent with this, it also binds FKBP, but at a K_D 20-fold higher than SLF-CR.

Crosslinking and Electrophoresis: Solutions containing 25 μM Aβ(1-42), 1 μM FKBP, and 1 μM drug in PBS were agitated for 3 hours at 37 °C. Photocrosslinking was performed according to the methods of Bitan et al. (*S5*). Crosslinked samples were separated on 4-12 % Bis-Tris gradient gels (Pierce) with 1x MES buffer and proteins stained with the Silver Stain Plus kit (BioRad).

Supporting Online Material: Text

For the bifunctional molecule, SLF-CR (Fig. S1A), to recruit FKBP to Aβ, this reagent must retain binding to Aβ via its CR moiety and to FKBP via its SLF moiety. As a measure of SLF-CR function, therefore, we examined the binding of the compound to immobilized Aβ(1-40) by surface plasmon resonance (*S3, S6*). The affinity of SLF-CR for Aβ was not significantly different from that of CR, which suggests that the

conjugation of SLF to congo red does not dramatically interfere with binding (Fig. S1B). Similarly, unmodified SLF and SLF-CR bound immobilized FKBP with comparable affinity (Fig. S1C). Thus, the targeting and recruiting domains of SLF-CR retained affinity for their respective targets.

To test the capacity of SLF-CR to bind Aβ and FKBP simultaneously, the bifunctional compound was pre-incubated with FKBP and the resulting solutions passed over immobilized amyloid. In surface plasmon resonance, the response (in arbitrary units, RUs) is proportional to the mass of material assembled on the surface (*S6*). We used this characteristic to determine whether SLF-CR could form a ternary complex with FKBP and immobilized Aβ. The combination of SLF-CR and FKBP gave a greater response (~230 RUs) than SLF-CR alone (~140 RUs) (Fig. S1D), suggesting that SLF-CR can trigger formation of the FKBP/drug/Aβ ternary complex.

Interestingly, SLF-CR/FKBP demonstrated only a slightly enhanced affinity for Aβ when compared to SLF-CR alone (Fig. S1). This result suggests that there are minimal favorable or unfavorable protein-protein contacts between FKBP and Aβ in the ternary complex. A one-to-one binding equation was used to calculate the K_D in parts C and D. The discrepancy between the K_D values in parts B and D, which is particularly apparent when CR is used as the analyte, is a result of the simplification used in part D to yield a single dissociation constant. These results suggest that any improvements in efficacy are not derived from enhanced affinity and may, instead, be a function of recruited steric bulk.

The chaperone activity of FKBP is to catalyze the cis-trans isomerization of proline residues. Therefore, we considered that the enzymatic activity of FKBP could, in addition to steric bulk, contribute to inhibitory potency. To examine this possibility,

we added progressively higher molar concentrations of recombinant FKBP to solutions of Aβ and monitored fibrillogenesis by thioflavin T fluorescence. We expected that, if isomerase activity provides inhibitory potency, high bulk solution levels of FKBP would mimic the localized recruitment by bifunctional molecules. Thioflavin T results strongly suggested that enzymatic activity was insufficient for inhibiting aggregation (Fig. S2A). This result does not imply that enzymatic activity never influences aggregation or folding of peptides involved in neurodegeneration. For example, while Aβ lacks prolines, other proteins implicated in Alzheimer's disease, such as microtubule-binding protein tau, have a number of proline residues.

Based on the results from the TEM and AFM experiments (see Fig. 2), the complex between SLF-CR and FKBP prevents fibril formation by trapping an intermediate along the Aβ aggregation pathway. To better characterize this intermediate, we examined the distribution of Aβ oligomers by electrophoretic analysis using a crosslinking technique developed for use with Aβ by the Teplow group (*S5*). Oligomers ranging from dimers to hexamers were routinely observed by this method (Fig. S2B). Addition of CR/FKBP decreased the relative intensity of oligomer bands compared to the untreated control. The SLF-CR/FKBP-treated samples, however, contained a predominance of tetramers. Although these results do not implicate tetramers as the sole trapped intermediate, they do suggest SLF-CR/FKBP functions by a different mechanism than congo red.

Supporting Online Material: Figure Legends

Fig. S1. Synthesis of SLF-CR and SPR analysis. (**A**) Routine synthetic methods were used to generate SLF-CR. Briefly, EDC/NHS-mediated amide bond formation

between the free acid of SLF and an excess of CR (Sigma, St. Louis, MO) in *N,N*-dimethylformamide (DMF) was carried out with stirring for 2-3 hours at 22 °C. The reaction mixture was subject to silica gel chromatography (10:90 MeOH:ethyl acetate) and SLF-CR purified to ~80% (estimated from TLC). Based on mass spectrometry, the major impurity was uncoupled congo red. The expected mass of SLF-CR was confirmed by ESI mass spectrometry (theoretical M^{+2Na}: 1261.3; observed M^{+2Na}: 1260.9). (**B-D**) Binding constants of drugs (± SEM) for immobilized Aβ and FKBP, as determined on a BIAcore 2000 instrument (*S3*). Schematics illustrate the identity of the surface and the injected analytes. The ΔRUs were determined by measuring the absolute response in the presence of drugs/protein and subtracting the reference signal. This value is proportional to the mass of drug/FKBP accumulated on the surface. Aβ(1-40) was prepared by resuspending peptide in DMSO to 100 μM. Fresh solutions were diluted to 25% DMSO with HBS-EP (BIAcore) and immediately immobilized. FKBP12 was immobilized in 10 mM sodium acetate pH 5.0 at a protein concentration of 10 μM using EDC/NHS. SPR solutions were prepared with appropriate solvent control (ethanol) and injected over the CM5 chip described in the main text. Injections were KINJECTS of 40 μL at a flow rate of 10 μL/min. Report points were obtained at 90% of the injection. Data from two separate injections were averaged, the values of control lane injections subtracted and the error expounded. The resulting values were plotted as a function of analyte concentration. Drug concentrations were determined by mass and confirmed by UV spectroscopy. Concentration of recombinant FKBP was determined by Bradford assay, using BSA as a standard. Binding isotherms were fit in PRISM 3.0 (GraphPad Software, San Diego, CA).

Fig. S2. Evaluation of bifunctional inhibitor SLF-CR. (**A**) Thioflavin T reactivity was used to measure the aggregation of Aβ, as described in the main text. The inhibitor of FKBP, FK506, was added to 25 μM. (**B**) Aβ aggregates in samples treated with FKBP alone, CR/FKBP or SLF-CR/FKBP. The number of Aβ peptides included in the aggregates were estimated from internal size standards and shown on the left. The band between the tetramer and pentamer is recombinant FKBP. (**C**) MTT viability assay was performed as per Figure 3 of the main text. Results are the average (± SEM) of triplicate wells and are representative of three experiments. Aβ(1-42) was used at 25 μM and FKBP was at 1 μM. (**D**) Quantitation of total number of cells per field (average of 5 fields ± SEM) as described in Fig. 3C.

Fig. S3. Synthesis and evaluation of bifunctional inhibitors in the linker series. (**A**) The methods used to generate the linker series compounds were similar to those used to create SLF-CR. Briefly, amine-bearing linkers were coupled to SLF by EDC/NHS methods and purified by silica gel chromatography. The resulting SLFs with installed linkers and free carboxylates were coupled to CR, purified, and characterized by ESI mass spectrometry: SLF-Gly-CR (theoretical M^{+2Na}: 1273.39; observed M^{+2Na}: 1273.2), SLF-But-CR (theoretical M^{+2Na}: 1315.47; observed M^{+2Na}: 1314.8), SLF-Benz-CR (theoretical M^{+2Na}: 1335.46; observed M^{+2Na}: 1336.0). (**B**) Inhibition of Aβ aggregation by bifunctional inhibitors in the absence of FKBP. Note that, in the absence of drug, all the compounds have similar efficacy. The SLF-Benz-CR/FKBP data are shown for comparison. Results are the average (± SEM) of at least two experiments performed in triplicate. (**C**) Dose dependent detoxification of Aβ by compounds in the linker series. FKBP was added to 1 μM in all cases.

Supporting Online Material: References

S1. A. S. Crystal, *et al*., *J. Neurochem.* **86**, 1359 (2003).

S2. I. A. Graef, *et al*., *Nature* **401**, 703 (1999).

S3. C. W. Cairo, A. Strzelec, R. M. Murphy, L. L. Kiessling, *Biochemistry* **41**, 8620-8629 (2002).

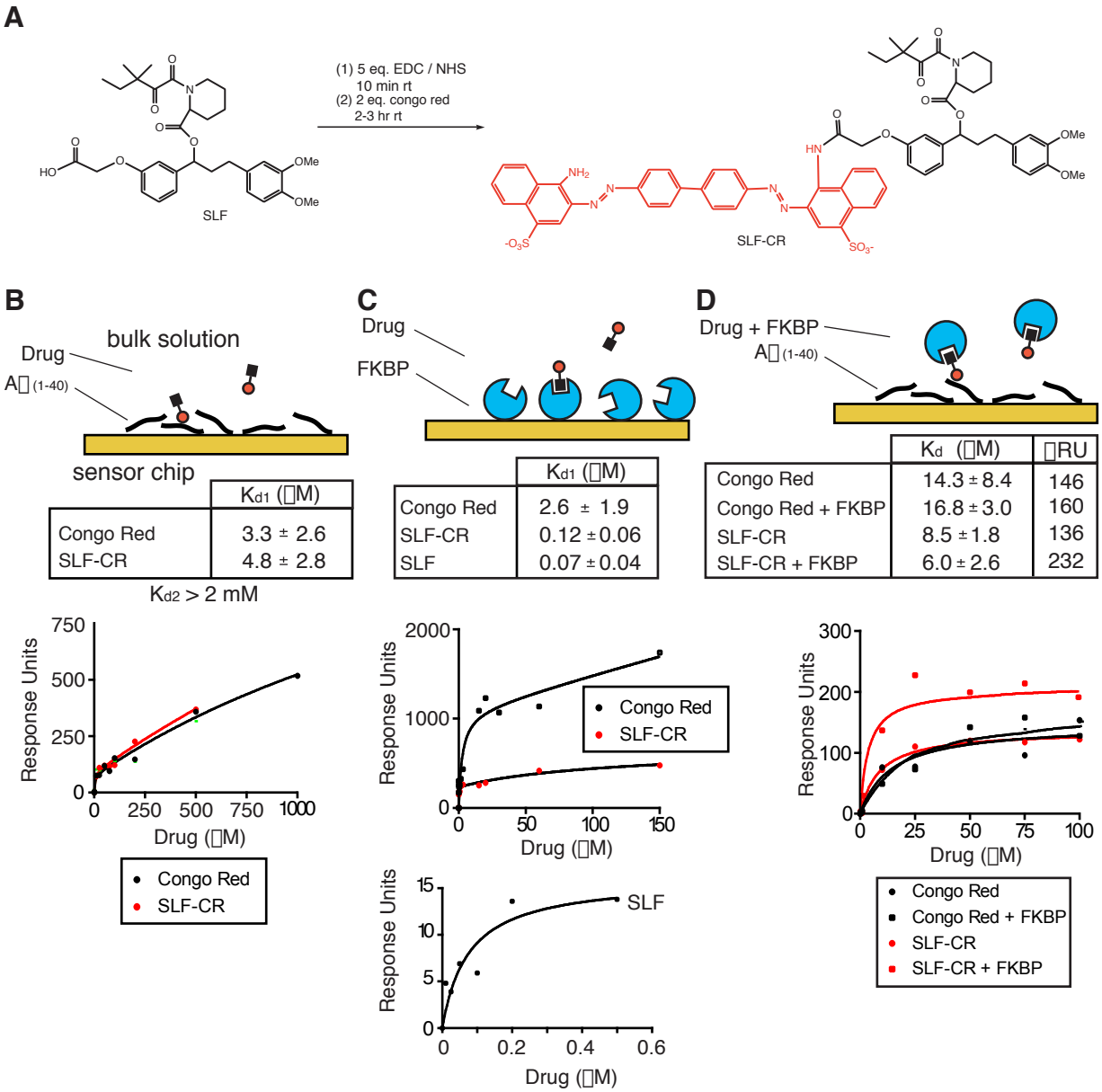
S4. R. Khurana, V. N. Uversky, L. Nielsen, A. L. Fink, *J. Biol. Chem.* **276**, 22715 (2001).

S5. G. Bitan, *et al*. *Proc. Natl. Acad. Sci.* **100**, 330 (2003).

S6. D. G. Myszka, *J. Mol. Recognit.* **12**, 279-284 (1999).

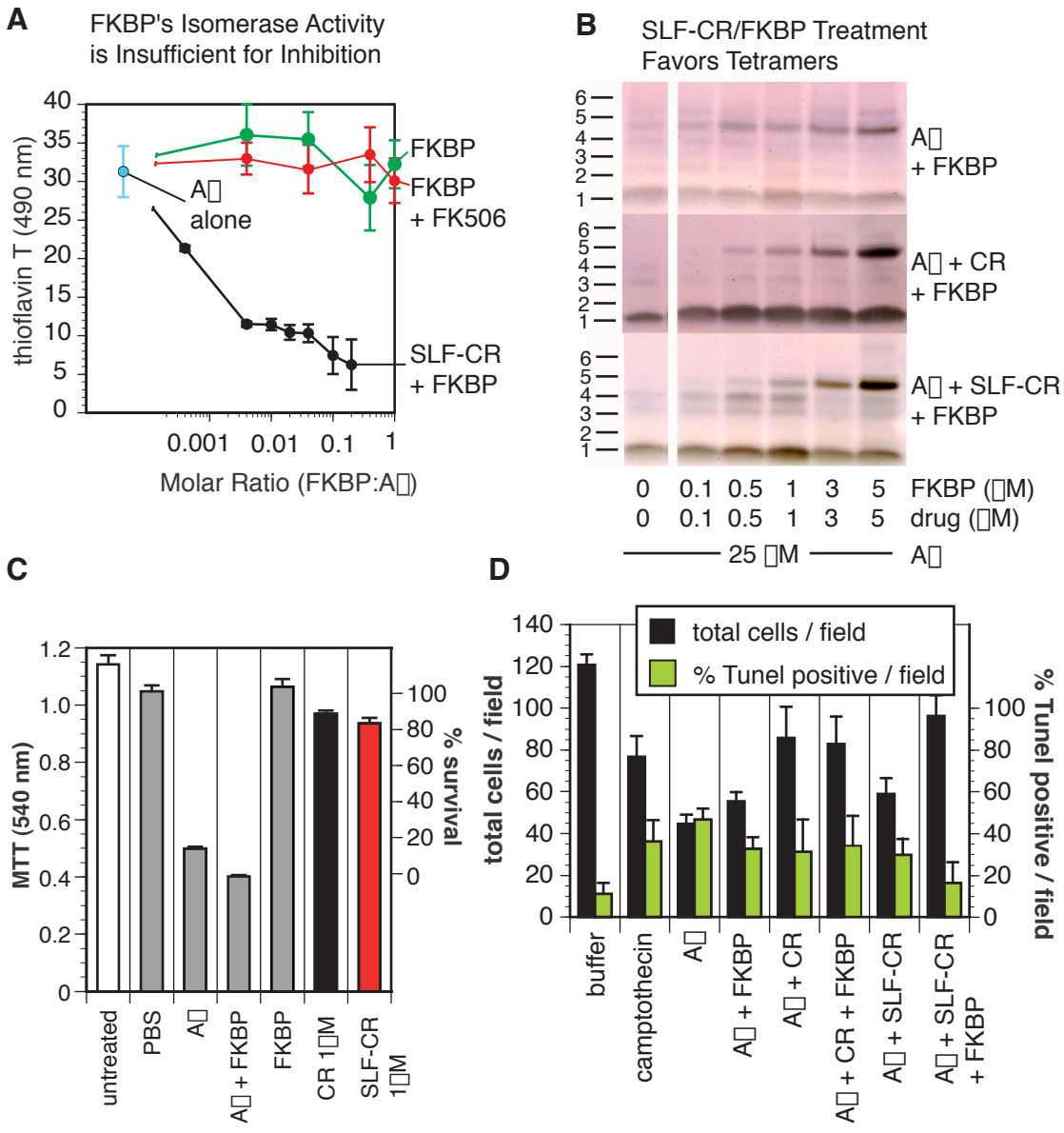
S7. Microscopy was performed in the Stanford Cell Sciences Imaging Facility.

Supplemental Fig. S1. Synthesis of SLF-CR and SPR analysis.



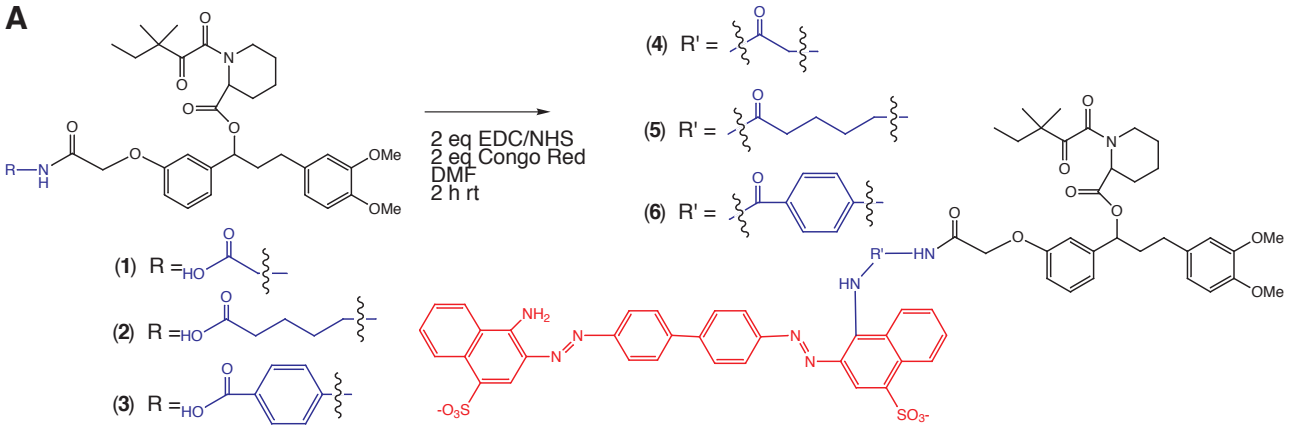
Gestwicki, Crabtree, and Graef

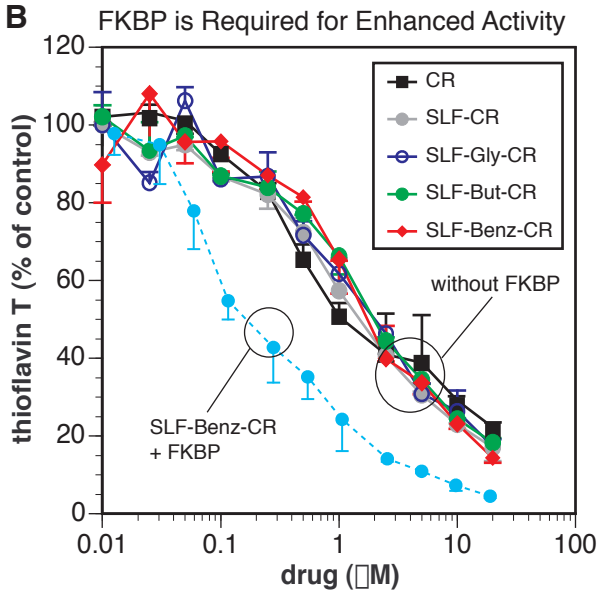
Supplemental Fig. S2. Evaluation of bifunctional inhibitor SLF-CR.

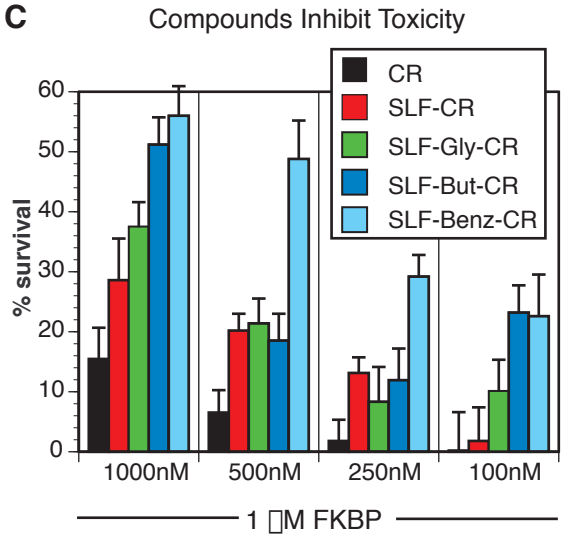


Gestwicki, Crabtree, and Graef

Supplemental Fig. S3. Synthesis and evaluation of bifunctional compounds in the linker series.







Gestwicki, Crabtree, and Graef