Supplementary Material (ESI) for Molecular BioSystems

Supporting Information

Screening of a branched peptide library with HIV-1 TAR RNA

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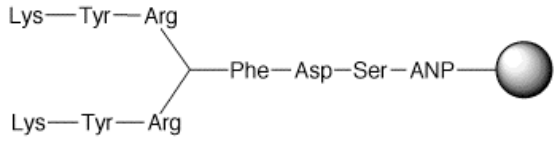
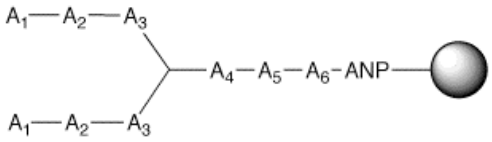
Abbreviations:

DCM: Dichloromethane
DMF: Dimethylformamide
MeOH: Methanol
ANP: 3-Amino-3-(2-nitrophenyl)propionic acid
HCTU: 2-(6-Chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminiumhexafluorophosphate
DIEA: Diisopropylethylamine
NMP: N-Methylpyrrolidinone
TFA: Trifluoroacetic acid
TIS: Triisopropylsilane
EDT: 1,2-ethanedithiol

1. Synthesis of branched peptides on resin.

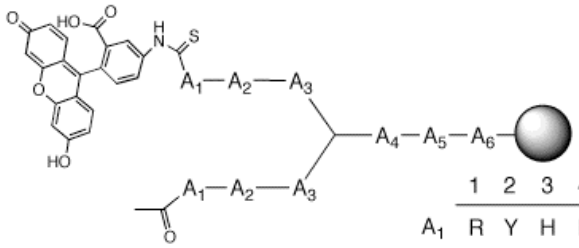


Control Branched Peptide $(KYR)_2$*FDS



3.3.3. Library (6 variable positions)

A_1	A_2	A_3	A_4	A_5	A_6
Asp	Arg	Tyr	His	Tyr	Phe
Arg	Asn	Leu	Val	Arg	Leu
Tyr	His	Trp	Asn	Ala	Asp
His	Val	Ala	Trp	Gln	Ser



FITC-Peptide

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
A_1	R	Y	H	R	R	R	R	Y	R	D	R	R	R	R	R	R	R
A_2	R	R	R	R	R	R	R	R	R	N	R	R	R	R	R	R	R
A_3	L	A	W	W	L	Y	W	L	A	L	A	W	Y	Y	A	L	L
A_4	W	H	W	H	N	V	H	W	N	H	V	H	N	V	V	H	H
A_5	Y	R	A	A	R	R	Y	R	Y	Y	Y	A	Q	Q	R	Y	R
A_6	L	F	S	L	F	L	D	L	F	F	F	S	D	L	D	L	F

Synthesis of the branched peptides was achieved by solid phase peptide synthesis using N-α-Fmoc protected L-amino acids (Novabiochem), HCTU (Peptides International) in DMF as coupling reagent, and DIEA (Aldrich). Tentagel Macrobead-NH_2 resin (Peptides International) with 0.3 mmol/g loading was coupled with photocleavable linker Fmoc-ANP[1] before synthesizing the control branched peptide $(KYR)_2$*FDS and the 3.3.3 library. Solid phase synthesis was done on a vacuum manifold (Qiagen) outfitted with 3-way Luer lock stopcocks (Sigma) in either Poly-Prep columns or Econo-Pac polypropylene columns (Bio-Rad). The resin was mixed in solution by bubbling argon during all coupling and washing steps.

Coupling ANP and N-α-Fmoc protected L-amino acids in control branched peptide:

Tentagel Macrobead-NH_2 resin (28 mg) with 0.3 mmol/g loading (Peptides International) was swollen in DCM (2 mL, 2 x 15 min) followed by DMF (2 mL, 15 minutes). Tentagel Macrobead-NH_2 resin was first coupled for 2 hours in DMF with 3 equiv. of Fmoc-ANP in presence of HCTU (3 equiv) and DIEA (5 equiv). N-Fmoc amino acids (3 equiv) were then coupled to resin bearing ANP linker in DMF in the presence of HCTU (3 equiv) and DIEA (5 equiv). Fmoc-Lys(Fmoc)-OH was used as the branching unit. Molar equivalences of all reagents were doubled in coupling reactions

after incorporation of the branching unit. Beads were washed extensively with DMF between reactions, and were tested for completeness via Kaiser test.

Coupling ANP and N-α-Fmoc protected L-amino acids in 3.3.3 library:

Tentagel Macrobead-NH_2 resin (200 mg) with 0.3 mmol/g loading was swollen and coupled with ANP as was done in the preparation of the control branched peptide. N-Fmoc amino acids (3 equiv) were then coupled to resin bearing ANP linker in NMP in the presence of HCTU (3 equiv) and DIEA (5 equiv). Molar equivalences of reagents were doubled after incorporation of the Lys branching unit. The standard split and pool method was utilized between reactions in the preparation of the 3.3.3 library. Beads were washed extensively with NMP between reactions, and were tested for completeness via Kaiser test.

Coupling N-α-Fmoc protected L-amino acids in FITC-peptides:

Rink amide MBHA resin (40 mg) with 0.7 mmol/g loading (Novabiochem) was swollen in DCM (2 mL, 2 x 15 min) followed by DMF (2 mL, 15 minutes). N-Fmoc amino acids (3 equiv) were then coupled directly to resin in DMF in the presence of HCTU (3 equiv) and DIEA (5 equiv). Fmoc-Lys(ivDde)-OH (Novabiochem) was used as the branching unit. The orthogonally protected Lys was selectively deprotected with 20% piperidine, and the first branch was synthesized with an acetyl cap at the N-terminus. Capping was accomplished by treating the Fmoc deprotected terminal amino acid with 1:1 acetic anhydride:DMF for 30 minutes. The ivDde protecting group was then removed with 2% hydrazine in DMF (2 mL, 2 x 5 min). The second branch was then synthesized, and the final amino acid was left Fmoc protected. The resin was washed with DMF, DCM, and MeOH before being dried and stored in a desiccator. Beads were washed extensively with DMF between reactions, and were tested for completeness via Kaiser test.

Coupling with fluorescein-5-isothiocyanate in FITC-peptides:

Once peptide synthesis was complete on the Rink amide MBHA resin, the uncapped N-terminus of the branched peptides was Fmoc deprotected and washed extensively with DMF. The peptides were then fluorescently labeled by mixing the resin with 6 equiv of fluorescein-5-isothiocyanate (Sigma) and DIEA (14 equiv) in minimal DMF for 12 hours in the dark. The resin was then washed with DMF, DCM, and MeOH. The beads were dried and immediately treated with 94:1:2.5:2.5 TFA/TIS/H_2O/EDT (v/v).

Cleavage of Fmoc protecting groups:

N-α-Fmoc protected amino acids were deprotected by mixing the resin in a 20% solution of piperidine in DMF (2 ml, 2 x 10 min). Resin was washed extensively with DMF after filtering off cleaving solution.

Side-chain deprotection and peptide cleavage with 94% TFA, 1% TIS, 2.5% H_2O, 2.5% EDT:

The amino acid side-chains were deprotected by a 3-hour treatment with 94:1:2.5:2.5 TFA/TIS/H_2O/EDT (v/v). After deprotection, resin was washed extensively with DMF, DCM, and MeOH before drying and storing it at -20 $^{\circ}$C protected from the

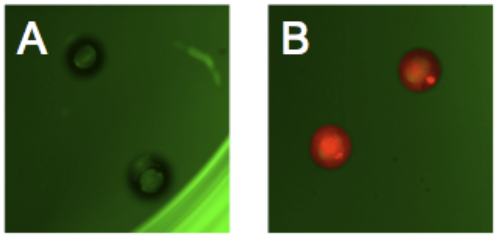
light. Treatment of Rink amide MBHA resin with the 94% TFA cocktail also afforded cleavage of the peptide when preparing the FITC-peptides. After peptide cleavage, TFA was removed under reduced pressure. The resulting yellow peptide was washed several times with cold diethyl ether and was finally dried to a yellow powder overnight under nitrogen flow. Dried peptide was stored at -20 °C in the dark until it was purified.

2. Control Experiments with branched peptide $(KYR)_2$*FDS

DY557 fluorescence intensity over auto-fluorescence of Tentagel Macrobead-NH_2 resin:

Due to the auto-fluorescence displayed by the Tentagel Macrobead-NH_2 resin, the ability to visualize the fluorescence of DY547 labeled HIV-1 TAR RNA (5'- DY547-GCC-CGA-UUU-GAG-CCU-GGG-AGC-UCU-CGG-GC-3', Dharmacon) needed to be insured. The control branched peptide $(KYR)_2$*FDS was incubated in 1 μM DY547 labeled HIV-1 TAR RNA for 1 hour in phosphate buffer (0.2 M sodium phosphate, pH 7.45 + 10 mM NaCl). These beads were then washed extensively with the same buffer before examining them in a sterile 96 well plate (Nunc), with a Zeiss LSM 510 microscope set to longpass 585. The fluorescence intensity of these RNA treated beads was compared with the auto-fluorescence of untreated control branched peptide resin, and the detector sensitivity was adjusted to remove auto-fluorescence of the resin as shown in Figure S1. This study showed that resin bound with DY547 labeled HIV-1 TAR RNA could be distinguished from the auto-fluorescence of the resin.

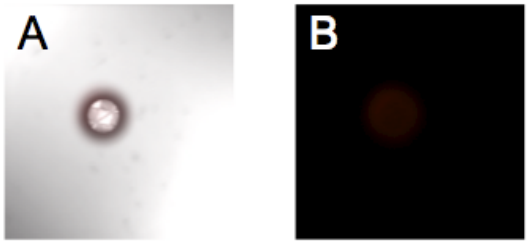
Figure S1. DY547 fluorescence intensity over auto-fluorescence of Tentagel Macrobead-NH_2 resin. A: Control branched peptide $(KYR)_2$*FDS without treatment with 1 μM DY547 labeled HIV-1 TAR RNA. B: Control branched peptide $(KYR)_2$*FDS with treatment of 1 μM DY547 labeled HIV-1 TAR RNA for 1 hour in phosphate buffer.



Organic washing conditions to remove bound RNA:

Control branched peptide with bound DY547 HIV-1 TAR RNA (shown in Figure S1-B) was washed with DMF (5x) and MeOH (5x) in order rinse away the fluorescent RNA. The washed resin was then examined by confocal microscopy in a 96 well plate. Reduced fluorescence was observed after this procedure as is seen in Figure S3. This procedure was also used to remove RNA from screened hits in the 3.3.3 library prior to the second round of screening, and prior to photocleavage of branched peptides from the resin.

Figure S3. Organic washed control branched peptide $(KYR)_2$*FDS. A: Washed resin shown as transmitted light + fluorescence image. B: Washed resin shown as fluorescence image only.



Optimizing screening conditions with control branched peptide $(KYR)_2$*FDS:

Screening conditions were optimized using the control branched peptide $(KYR)_2$*FDS. About 2000 beads were divided evenly among 6 non-stick 1.5 mL microfuge tubes. All incubations were performed in 300 µL of TK buffer (50 mM Tris•HCl, pH 7.4/ 20 mM KCl/ 0.1% Triton X-100) at 4 °C. A Barnstead/Thermolyne Labquake rotisserie shaker was used to agitate the incubation mixtures. The first set of 3 controls was done in two incubation steps. The first incubation was 3 hours and the components are shown in Table 1. The beads were then washed 3 times with TK buffer before being incubated in 3.4 nM DY547 labeled HIV-1 TAR RNA for 3 hours. The second set of 3 controls incubated for 6 hours at 4 °C with the components shown in Table 2. Alpha-synuclein mRNA was prepared by standard cloning and transcription procedures. Bovine serum albumin (BSA) was obtained from New England BioLabs Inc.

Table 1:

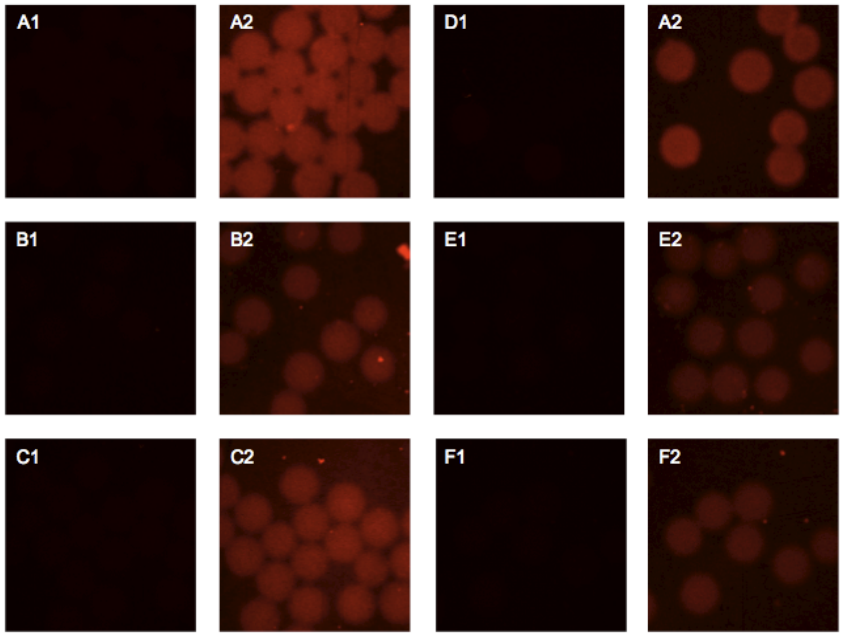
First Incubation Components	Control-A	Control-B	Control-C
1 mg/ml BSA	X		X
0.5 mM alpha-synuclein mRNA		X	X

Table 2:

Incubation Components	Control-D	Control-E	Control-F
1 mg/ml BSA	X		X
0.5 mM alpha-synuclein mRNA		X	X
3.4 nM HIV-1 TAR RNA	X	X	X

After the final incubation, a Zeiss LSM 510 microscope set to longpass 585 was used to image the beads. The most stringent conditions were found to be when the resin was incubated simultaneously with DY547 HIV-1 TAR RNA, BSA, and alpha-synuclein mRNA (Figure S2).

Figure S2. Optimizing screening conditions with control branched peptide. A1-F1: Images of beads from different incubation conditions. A2-F2: Same as A1-F1 with detector sensitivity adjusted for better visualization.



3. Screening of the 3.3.3 library for binding to HIV-1 TAR

Initially, ~2000 beads of the 3.3.3 library were taken into a 1.5 µl non-stick microfuge tube. The beads were allowed to mix for 3 hours with 1 mg/mL BSA and 0.5 µM alpha-synuclein mRNA in 500 µl of TK buffer at 4 °C. After the first incubation, the beads were washed (5x) with TK buffer and were then incubated for 3 hours at 4$^{\circ C}$ in 500 µl of 3.4 nM DY547 HIV-1 TAR mRNA in TK buffer. The beads were then washed (5x) with TK buffer before being evenly distributed among the wells of a 96 well plate and analyzing them by confocal microscopy. This initial screening procedure yielded 6 beads with elevated fluorescence over background (Figure S3, A-E). These first 6 hits were rinsed with the organic wash and stored at 4 °C until they were photocleaved and sequenced by MALDI-TOF.

When screening the remaining beads of the 3.3.3 library, more stringent conditions were used in an attempt to reduce the total number of hit beads. The first incubation proceeded as done previously, but the second incubation time was limited to 1 hour. This procedure yielded an additional 10 beads with elevated fluorescence (Figure S3, F-N). These beads were rinsed with the organic wash and were then re-screened under even more stringent conditions.

The re-screening procedure was comprised of two incubations. The 10 hits were first mixed for 3 hours in 1 mg/mL BSA and 0.5 µM alpha-synuclein mRNA in 300 µl of TK buffer at 4 °C. The beads were then extensively washed with TK buffer before incubating them a second time for 30 minutes in 300 µl of TK buffer with 3.4 nM DY547 HIV-1 TAR mRNA at 4 °C. The beads were extensively rinsed with TK buffer prior to imaging them in a 96 well plate with the confocal microscope (Figure S4). The re-

screened beads were finally treated by the organic wash to remove any bound RNA prior to photocleavage and MALDI-TOF sequencing.

Figure S3: The 16 hits screened from the 3.3.3 library. A-E: Initial 6 hits. F-N: Additional 10 hits found with more stringent screening conditions.

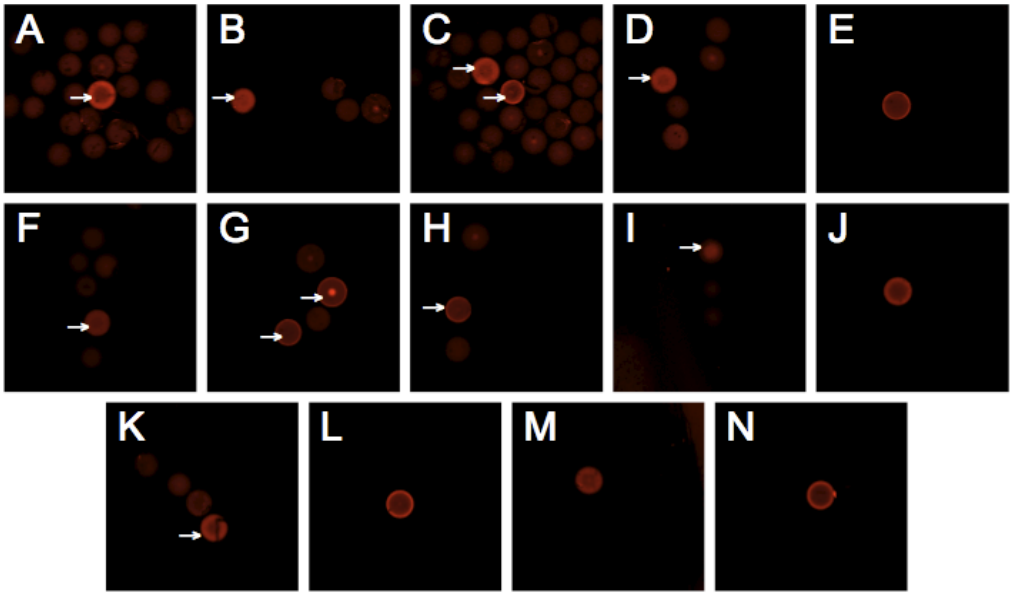
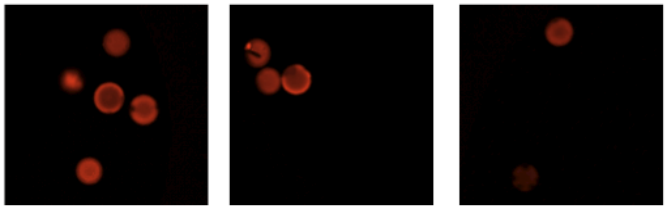


Figure S4: The 10 hits from the 3.3.3 library, which were re-screened under stringent screening conditions.



4. Photocleavage of peptides from the ANP resin and MALDI-TOF sequencing

The 3.3.3 library peptides and control peptides were released from the resin by photocleavage of the ANP photolinker. Beads selected for photocleavage were taken up individually into clear non-stick 0.5 mL microfuge tubes with 20 µL of 1:1 MeOH:H_2O. The microfuge tubes containing resin were placed into a foil-lined container and were irradiated for 1 hour with light at 365 nm in using a 4 W handheld UV lamp. The resin was immediately removed from the supernatant after photocleavage. The peptide sequences were obtained from MALDI-TOF MSMS.

5. Synthesis, HPLC purification and MALDI-TOF identification of FITC-peptides (BP15-BP17, T15)

Branched peptides 15-17 were resynthesized as described above with a single FITC label to be used in fluorescence polarization experiments. A linear peptide, T15 RRAGVRD, was also synthesized using Rink Amide MBHA resin utilizing standard Fmoc chemistry. T15 was also coupled to FITC as described above. Each synthesized

FITC-peptide was purified by HPLC. Fractions containing the peptide of interest were identified by MALDI-TOF MS.

MALDI/MS:
FITC-BP15: FITC-$(RRA)_2$*VRD; Calculated: 1712.83, Found: 1713.86
FITC-BP16: FITC-$(RRL)_2$*HYL; Calculated: 1840.93, Found: 1841.07
FITC-BP17: FITC-$(RRL)_2$*HRF; Calculated: 1867.95, Found: 1868.99
T15: FITC-RRAGVRD; Calculated: 1216.52, Found: 1217.70

6. Fluorescence polarization experiments

Fluorescence polarization experiments were conducted to obtain K_d values using the FITC-peptides that had been HPLC purified and identified by MALDI-TOF MS. Experiments were conducted in black, flat-bottom 384 well plates (Corning) and an Analyst AD (LJL BioSystems). The FITC-peptides (0.5 nM) were individually titrated with HIV-1 TAR RNA (up to 100 μM) used during the on-bead assay or mutTAR (U24→C24, synthesized in-house using a Mermade IV oligonucleotide synthesizer) at 25°C in a buffer containing 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 1 mM $MgCl_2$, 5 mM KCl, and 140 mM NaCl at pH 7.4. Samples were excited at 485 nm and emission was monitored at 530 nm. Each data point was an average of 5 scans, and all fluorescence polarization experiments were conducted in triplicate.

Reference:

(1) D. S. Tan, M. A. Foley, B. R. Stockwell, M. D. Shair, S. L. Schreiber, Synthesis and preliminary evaluation of a library of polycyclic small molecules for use in chemical genetic assays, *J. Am. Chem. Soc.,* 1999, **121**, 9073-9087.