Supplementary Material (ESI) for Medicinal Chemistry Communications

Supplementary Information

Affinity Selection and sequence-activity relationships of HIV-1 membrane fusion inhibitors directed at the drug-resistant variants

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Standard protocol for peptide synthesis. Protected peptide-resins were manually constructed by Fmoc-based solid-phase peptide synthesis. *t*-Bu ester for Asp and Glu, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg, *t*-Bu for Thr, Tyr and Ser, Boc for Lys, Trt for Gln, Asn, and His were employed for side-chain protection. Fmoc-amino acids were coupled using five equivalents of reagents [Fmoc-amino acid, *N*,*N*'-diisopropylcarbodiimide (DIC), and HOBt·H_2O] to free amino group in DMF for 1.5 h. Fmoc deprotection was performed by 20% piperidine in DMF (2 × 1 min, 1 × 20 min). For inhibitory C-HR peptides, the N-terminus of the peptides was acetylated by treatment with Ac_2O (10 equiv.) and pyridine (10 equiv.) in DMF. The resulting protected resin was treated with TFA/thioanisole/*m*-cresol/1,2-ethandithiol/H_2O (80:5:5:5:5, 1.25 cm^3 per 100 mg resin) at room temperature for 2 hr. After removal of the resin by filtration, the filtrate was poured into ice-cold dry diethyl ether. The resulting powder was collected by centrifugation and washed with ice-cold dry diethyl ether. The crude product was purified by preparative HPLC on a Cosmosil 5C18-ARII preparative column (Nacalai Tesque, 20 × 250 mm, flow rate 10 cm^3 min^{-1}) to afford the expected peptides. All peptides were characterized by MALDI-TOF-MS (AXIMA-CFR plus, Shimadzu, Kyoto, Japan), and the purity was calculated as >95 % by HPLC on a Cosmosil 5C18-ARII analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1 cm^3 min^{-1}) at 220 nm absorbance.

General procedure for the split-mix synthesis of the peptide mixture: synthesis of $SC35EK_{S138X}$ derivatives. Protected peptide resin for SC35EK(139–151) was constructed at a 0.40 mmol scale on NovaSyn® TGR resin (0.26 mmol g^{-1}) using a standard protocol. The resin was split into 24 portions (approximately 0.017 mmol each), and each fraction was coupled with one of the natural amino acids for position 138. Half of each fraction (approximately 0.0083 mmol) was combined into one portion, which was subjected to further coupling steps for the SC35EK(117–137) sequence. The other resin potions were utilized for the parallel synthesis of each peptide. After acetylation of the N-terminus, final deprotection of the resin (300 mg) and HPLC purification using a standard protocol provided the expected peptide library (41.6 mg) with a single substitution at position 138.

Affinity selection-mass spectrometry experiments. A pool of C-HR peptides [19 mmol dm^{-3} total in 1 cm^3 of incubation buffer (25 mmol dm^{-3} HEPES, 100 mmol dm^{-3} NaCl, pH 7.4)] were incubated for 2 hour at room temperature in the presence of the His-tagged N-HR peptide (10 mmol dm^{-3}). Ni-NTA agarose resin (Quagen, 0.030 cm^3) was added to the mixture and the solution was agitated for an additional two hours at room temperature. The resin was collected by centrifugation and washed with the incubation buffer (0.300 cm^3 × 2). After the captured peptides were treated with 50% AcOH (0.100 cm^3) for 30 min at room temperature, the eluent was analyzed by LC–MS (Alliance2695 HPLC coupled with QuattroMicro triple quadruple mass spectrometer, Waters). For the separation, a Cosmosil 5C18-ARII column (4.6 × 250 mm, Nacalai Tesque, Inc., Kyoto, Japan) was employed with a linear gradient of MeCN containing 0.1% (v/v) TFA at a flow rate of 1 cm^3 min^{-1} [30–40 % MeCN over 40 min for SC35EK derivatives; 35–45 % MeCN over 40 min for T-20EK derivatives]. The recovery rate of the captured C-HR peptides was calculated from the relative detected signals of $[M+3H]^{3+}$ and $[M+4H]^{4+}$ ions to the signals of each component in the parent C-HR mixture by LC–MS analysis in the single ion recording (SIR) mode.

Determination of drug susceptibility of HIV-1. The peptide sensitivity of infectious pNL4-3-derived clones was determined by the MAGI assay with some modifications.[S1] Recombinant HIV-1 clones carrying various mutations in gp41 of were employed for the assay.[S2] The target cells (HeLa-CD4-LTR-β-gal; 10^4 cells $well^{-1}$) were plated in 96-well flat microtiter culture plates. On the following day, the cells were inoculated with the HIV-1 clones (60 MAGI U $well^{-1}$, giving 60 blue cells after 48 h of incubation) and cultured in the presence of various concentrations of drugs in fresh medium. Forty-eight hours after viral exposure, all the blue cells stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were counted in each well. The activity of test compounds was determined as the concentration that blocked HIV-1 replication by 50% (50% effective concentration [EC_{50}]) of the dose-response curves from triplicate experiments.

Measurement of CD spectra. Peptides were dissolved in 5 mmol dm^{-3} HEPES buffer (pH 7.2) to a final concentration of 10 mmol dm^{-3}. The mixture of N-HR and C-HR peptides were incubated at 37 °C for 30 min prior to recording CD data. The wavelength-dependent molar ellipticity [θ] was monitored at 25 ºC as the average of 8 scans using a Jasco spectropolarimeter (Model J-710, Jasco

Inc., Tokyo, Japan). Thermal unfolding of the potential six-helical bundle in the presence of the N-HR peptide was monitored by the $[\theta]_{222}$ values over a temperature range of 4–90 °C at intervals of 0.5 °C after a 15-second equilibration at the desired temperature and an integration time of 1.0 sec. The midpoint of the thermal unfolding transition of each complex was defined as the melting temperature (T_m).

Table S1. Characterization data of synthetic SC35EK derivatives by MALDI TOF-MS.

X	$SC35EK_{S138X}$			$SC35EK_{Q141X}$		
	Formula (MH^+)	Calculated	Found	Formula (MH^+)	Calculated	Found
Ala	$C_{203}H_{326}N_{51}O_{65}$	4521.1	4520.7	$C_{201}H_{323}N_{50}O_{65}$	4480.0	4477.9
Asp	$C_{204}H_{326}N_{51}O_{67}$	4565.1	4564.9	$C_{202}H_{323}N_{50}O_{67}$	4524.0	4423.1
Glu	$C_{205}H_{328}N_{51}O_{67}$	4579.1	4578.2	$C_{203}H_{325}N_{50}O_{67}$	4538.0	4536.1
Phe	$C_{209}H_{330}N_{51}O_{65}$	4597.2	4596.6	$C_{207}H_{327}N_{50}O_{65}$	4556.1	4554.5
Gly	$C_{202}H_{324}N_{51}O_{65}$	4507.0	4506.7	$C_{200}H_{321}N_{50}O_{65}$	4466.0	4465.2
His	$C_{206}H_{328}N_{53}O_{65}$	4587.1	4587.0	$C_{204}H_{325}N_{52}O_{65}$	4546.1	4544.6
Ile	$C_{206}H_{332}N_{51}O_{65}$	4563.1	4563.1	$C_{204}H_{329}N_{50}O_{65}$	4522.1	4521.1
Lys	$C_{206}H_{333}N_{52}O_{65}$	4577.2	4577.7	$C_{204}H_{330}N_{51}O_{65}$	4537.1	4535.0
Leu	$C_{206}H_{332}N_{51}O_{65}$	4563.1	4563.0	$C_{204}H_{329}N_{50}O_{65}$	4522.1	4521.9
Met	$C_{205}H_{330}N_{51}O_{65}S$	4581.2	4580.5	$C_{203}H_{327}N_{50}O_{65}S$	4540.1	4539.4
Asn	$C_{204}H_{327}N_{52}O_{66}$	4564.1	4564.1	$C_{202}H_{324}N_{51}O_{66}$	4523.0	4522.8
Pro	$C_{205}H_{328}N_{51}O_{65}$	4547.1	4546.9	$C_{203}H_{325}N_{50}O_{65}$	4506.0	4505.2
Gln	$C_{205}H_{329}N_{52}O_{66}$	4578.1	4578.1	-[a]	-	-
Arg	$C_{206}H_{333}N_{54}O_{65}$	4506.2	4606.2	$C_{204}H_{330}N_{53}O_{65}$	4565.1	4564.2
Ser	$C_{203}H_{326}N_{51}O_{66}$[a]	4537.1	4536.3	$C_{201}H_{323}N_{50}O_{66}$	4496.0	4495.7
Thr	$C_{204}H_{328}N_{51}O_{66}$	4551.1	4550.8	$C_{202}H_{325}N_{50}O_{66}$	4510.0	4508.8
Val	$C_{205}H_{330}N_{51}O_{65}$	4549.1	4549.2	$C_{203}H_{327}N_{50}O_{65}$	4508.1	4507.9
Trp	$C_{211}H_{331}N_{52}O_{65}$	4636.2	4636.4	$C_{209}H_{328}N_{51}O_{65}$	4595.1	4594.6
Tyr	$C_{209}H_{330}N_{51}O_{66}$	4613.2	4611.5	$C_{207}H_{327}N_{50}O_{66}$	4572.1	4571.8

[a]Wild-type sequence.

Table S2. Characterization data of synthetic T-20EK derivatives by MALDI TOF-MS.

X	$T\text{-}20EK_{S138X}$			$T\text{-}20EK_{Q141X}$		
	Formula (MH^+)	Calculated	Found	Formula (MH^+)	Calculated	Found
Ala	$C_{213}H_{329}N_{52}O_{62}$	4610.2	4609.4	$C_{211}H_{326}N_{51}O_{62}$	4569.1	4568.3
Asp	$C_{214}H_{329}N_{52}O_{64}$	4654.2	4654.0	$C_{212}H_{326}N_{51}O_{64}$	4613.2	4612.6
Glu	$C_{215}H_{331}N_{52}O_{64}$	4668.2	4668.0	$C_{213}H_{328}N_{51}O_{64}$	4627.2	4626.5
Phe	$C_{219}H_{333}N_{52}O_{62}$	4686.3	4686.4	$C_{217}H_{330}N_{51}O_{62}$	4645.2	4644.6
Gly	$C_{212}H_{327}N_{52}O_{62}$	4696.2	4696.0	$C_{210}H_{324}N_{51}O_{62}$	4555.1	4555.1
His	$C_{216}H_{331}N_{54}O_{62}$	4676.3	4677.0	$C_{214}H_{328}N_{53}O_{62}$	4635.2	4635.3
Ile	$C_{216}H_{335}N_{52}O_{62}$	4652.3	4652.0	$C_{214}H_{332}N_{51}O_{62}$	4611.2	4611.0
Lys	$C_{216}H_{336}N_{53}O_{62}$	4667.3	4668.0	$C_{214}H_{333}N_{52}O_{62}$	4626.2	4625.3
Leu	$C_{216}H_{335}N_{52}O_{62}$	4652.3	4652.0	$C_{214}H_{332}N_{51}O_{62}$	4611.2	4611.3
Met	$C_{215}H_{333}N_{52}O_{62}S$	4670.3	4670.5	$C_{213}H_{330}N_{51}O_{62}S$	4629.3	4629.3
Asn	$C_{214}H_{330}N_{53}O_{63}$	4653.2	4654.0	$C_{212}H_{327}N_{52}O_{63}$	4612.2	4611.3
Pro	$C_{215}H_{331}N_{52}O_{62}$	4636.2	4636.0	$C_{213}H_{328}N_{51}O_{62}$	4595.2	4694.6
Gln	$C_{215}H_{332}N_{53}O_{63}$	4667.2	4668.0	-[a]	-	-
Arg	$C_{216}H_{336}N_{55}O_{62}$	4695.3	4696.0	$C_{214}H_{333}N_{54}O_{62}$	4654.3	4654.0
Ser	$C_{213}H_{329}N_{52}O_{63}$ [a]	4626.2	4625.3	$C_{211}H_{326}N_{51}O_{63}$	4585.1	4585.0
Thr	$C_{214}H_{331}N_{52}O_{63}$	4640.2	4640.0	$C_{212}H_{328}N_{51}O_{63}$	4599.2	4599.4
Val	$C_{215}H_{333}N_{52}O_{62}$	4638.2	4638.0	$C_{213}H_{330}N_{51}O_{62}$	4597.2	4596.6
Trp	$C_{221}H_{334}N_{53}O_{62}$	4725.3	4625.0	$C_{219}H_{331}N_{52}O_{62}$	4684.3	4684.0
Tyr	$C_{219}H_{333}N_{52}O_{63}$	4702.3	4702.2	$C_{217}H_{330}N_{51}O_{63}$	4661.2	4661.3

[a]Wild-type sequence.

Table S3. Characterization data of synthetic HR1 peptides by MALDI TOF-MS.

X	Formula (MH^+)	Calculated	Found
N36	$C_{186}H_{314}N_{57}O_{51}$	4164.8	4164.7
$N36_{V38A}$	$C_{184}H_{310}N_{57}O_{51}$	4136.8	4136.5
$N36_{N43D}$	$C_{186}H_{313}N_{56}O_{52}$	4165.8	4166.5
N54	$C_{264}H_{451}N_{80}O_{76}S_2$	6026.0	6026.0
$N54_{V38A}$	$C_{262}H_{447}N_{80}O_{76}S_2$	5998.0	5998.0
$N54_{N43D}$	$C_{264}H_{450}N_{79}O_{77}S_2$	6027.0	6026.3
$(His)_6$-N36	$C_{228}H_{368}N_{77}O_{58}$	5115.8	5115.7
$(His)_6$-$N36_{V38A}$	$C_{226}H_{364}N_{77}O_{58}$	5087.8	5088.6
$(His)_6$-$N36_{N43D}$	$C_{228}H_{367}N_{76}O_{59}$	5116.8	5116.7
$(His)_6$-N54	$C_{306}H_{505}N_{100}O_{83}S_2$	6977.0	6975.4
$(His)_6$-$N54_{V38A}$	$C_{304}H_{501}N_{100}O_{83}S_2$	6949.0	6948.7
$(His)_6$-$N54_{N43D}$	$C_{306}H_{504}N_{99}O_{84}S_2$	6978.0	6976.7

Figure S1. Representative data of CD analysis: (a) CD spectra of C-HR peptides in the presence of N-HR peptides. (b) Temperature dependence of the CD signal at 222 nm. T_m values were defined by the midpoint of the thermal unfolding transition state. Blue: N36–SC35EK complex, red: N54–T-20EK complex.

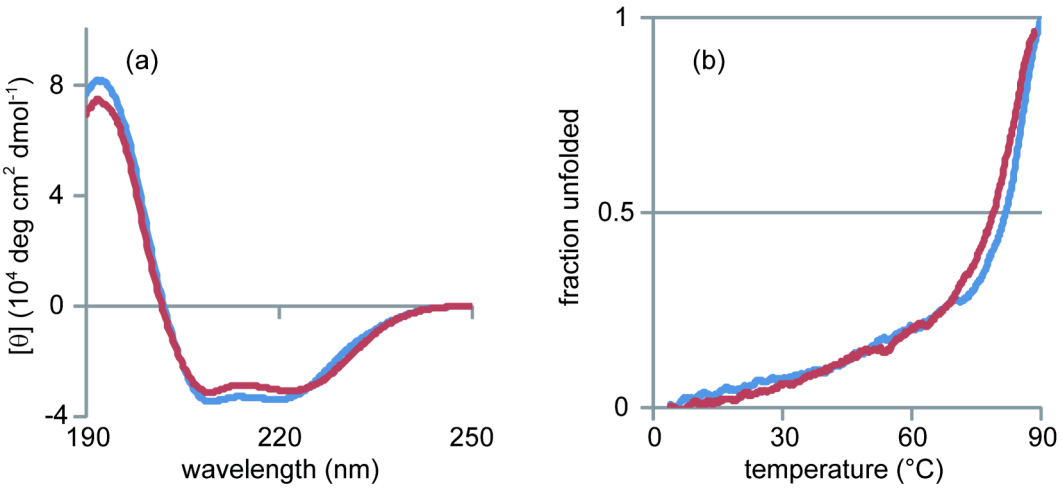


Table S4. Recovery rate in affinity selection and anti-HIV activity of $SC35EK_{S138X}$ and T-$20EK_{S138X}$ against enfuvirtide-resistant viruses.

X	$SC35EK_{S138X}$				$T\text{-}20EK_{S138X}$			
	HIV_{V38A}		HIV_{N43D}		HIV_{V38A}		HIV_{N43D}	
	Recovery (%)[a]	EC_{50} (nM)[b]	Recovery (%)[a]	EC_{50} (nM)[b]	Recovery (%)[a]	EC_{50} (nM)[b]	Recovery (%)[a]	EC_{50} (nM)[b]
Ser	11.8	1.1 ± 0.3	10.9	0.3 ± 0.1	9.3	3.4 ± 1.4	7.4	2.0 ± 0.4
Ala	11.4	0.7 ± 0.2	12.9	0.2 ± 0.1	10.0	2.2 ± 0.8	10.2	0.3 ± 0.1
Asp	0.7	310 ± 58	0.0	48 ± 9.9	1.4	>1000	2.2	>1000
Glu	5.9	270 ± 26	0.4	28 ± 10	2.2	>1000	2.8	560 ± 210
Phe	4.0	1.3 ± 0.5	3.4	0.2 ± 0.1	7.0	>1000	7.4	24 ± 5.4
Gly	11.0	1.0 ± 0.2	8.9	0.2 ± 0.1	7.8	10 ± 4.3	5.4	2.2 ± 0.6
His	3.4	22 ± 3.5	3.4	2.1 ± 0.4	3.7	250 ± 52	4.8	200 ± 34
Ile	10.3[c]	0.6 ± 0.1	12.9[c]	0.2 ± 0.1	10.1[c]	2.1 ± 0.5	10.8[c]	0.2 ± 0.1
Lys	0.0	500 ± 140	0.0	10 ± 3.5	1.1	>1000	1.3	500 ± 100
Leu	10.3[c]	0.8 ± 0.1	12.9[c]	0.2 ± 0.1	10.1[c]	3.4 ± 1.6	10.8[c]	0.2 ± 0.1
Met	11.5	0.7 ± 0.3	14.2	0.2 ± 0.1	9.9	1.8 ± 0.2	10.4	0.2 ± 0.1
Asn	2.7	3.6 ± 0.6	3.0	0.5 ± 0.2	2.9	>1000	3.0	30 ± 6.7
Pro	0.4	340 ± 61	0.3	130 ± 52	0.0	>1000	0.3	430 ± 160
Gln	2.0	8.5 ± 2.9	3.1	0.5 ± 0.2	2.3	>1000	3.2	49 ± 23
Arg	0.0	>1000	0.0	56 ± 7.0	1.7	>1000	2.1	>1000
Thr	10.8	0.8 ± 0.1	9.4	0.3 ± 0.1	8.1	28 ± 12	6.1	3.7 ± 0.1
Val	9.7	0.9 ± 0.3	12.3	0.2 ± 0.1	8.8	4.6 ± 0.5	8.8	0.3 ± 0.1
Trp	1.0	22 ± 5.6	2.4	2.7 ± 0.6	7.7	>1000	7.6	220 ± 17
Tyr	3.5	3.7 ± 0.8	2.7	0.7 ± 0.2	5.8	510 ± 150	6.2	40 ± 3.4

[a]The recovery rate was determined from the relative detected signals of $[M+3H]^{3+}$ and $[M+4H]^{4+}$ ions by LC–MS analysis. [b]EC_{50} was determined as the concentration that blocked HIV-1_{NL4-3} replication by 50% in the MAGI assay. To improve the replication kinetics, the D36G mutation, observed in the majority of HIV-1 strains, was introduced into the NL4-3 background used in this study. [c]Combined yield of S138I and S138L derivatives.

Figure S2. Correlation between anti-HIV activity of C-HR peptides and the thermal stability of potential six-helical bundles. (a) $SC35EK_{S138X}$, (b) $T\text{-}20EK_{S138X}$. Blue: wild-type, red: HIV_{V38A}, green: HIV_{N43D}.

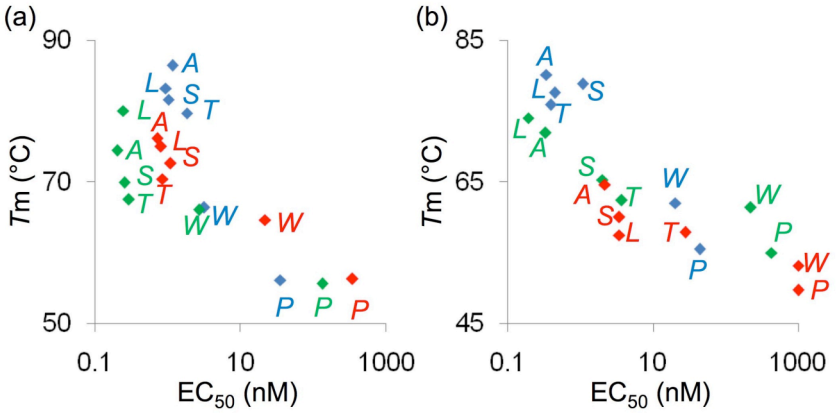


Figure S3. Correlation between the recovery rate of C-HR peptides in affinity selection and anti-HIV activity. (a) $SC35EK_{S138X}$ against HIV_{V38A}, (b) $SC35EK_{S138X}$ against HIV_{N43D}, (c) $T\text{-}20EK_{S138X}$ against HIV_{V38A}, (d) $T\text{-}20EK_{S138X}$ against HIV_{N43D}. R^2 values were calculated without using the data points of S138K/P/R mutants for (a) (n = 16), S138D/E/K/P/R mutants for (b) (n = 14), S138N/Q/H/W/D/E/K/P/R for (c) (n = 10), and S138D/P/R for (d) (n = 16).

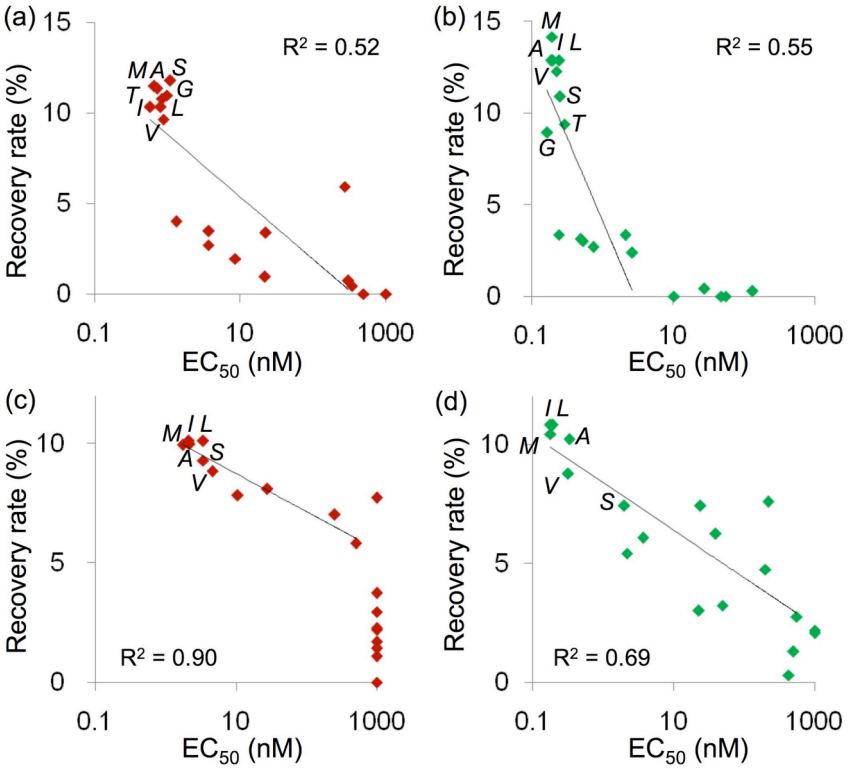
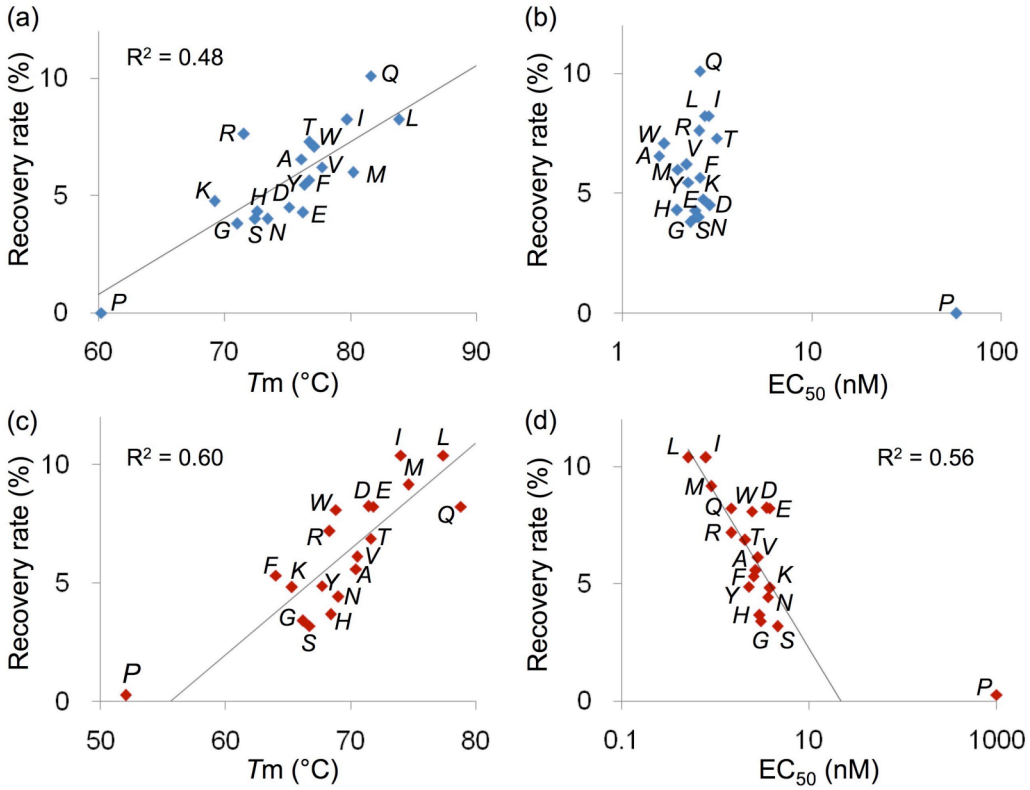


Figure S4. Correlation between the recovery rate of C-HR peptides in affinity selection and the thermal stability of potential six-helical bundles, or the anti-HIV activity. (a, b) $SC35EK_{Q141X}$, (c, d) $T\text{-}20EK_{Q141X}$. R^2 values were calculated without using the data points of S138P mutant (n = 18).



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S2 D. Nameki, E. Kodama, M. Ikeuchi, N. Mabuchi, A. Otaka, H. Tamamura, M. Ohno, N. Fujii and M. Matsuoka, *J. Virol.* 2005, **79**, 764-770.