Electronic Supplementary Material (ESI) for Molecular BioSystems.

Dihydrofolate Reductase Inhibitory Peptides Screened from Structured Design β-Loop Peptide Library Displayed on Phage

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Experimental section

General

All chemicals and solvents for peptide synthesis were of reagent or HPLC grade and were used without further purification. TARON resin was purchased from Clontech. Dihydrofolate (DHP), nicotinamide adenine dinucleotide phosphate (NADPH) and methotrexate (MTX) were purchased from Wako Pure Chemical Industries. Biotin-AC_5-OSu and 2-morpholinoethanesulfonic acid monohydrate (MES) were purchased from Dojindo. Dynabeads M-280 Streptavidin and Dynabeads M-280 Tosylactivated were purchased from Life Technologies. NeutrAvidin was purchased from Thermo Fisher Scientific. A β-loop peptide phage library was produced according to previous our method.[S1] RP-HPLC was performed on the Hitachi L7000 system using a COSMOSIL 5C18-ARII (ϕ10×250 mm) column for purification with a linear gradient of acetonitrile/0.1% trifluoroacetic acid (TFA) at a flow rate of 3.0 mL/min. ESI-MS was measured on a Shimadzu LCMS-2010.

Peptide synthesis

All peptides were synthesized by stepwise elongation techniques of Fmoc-protected amino acids on a TentaGel S RAM resin according to the Fmoc solid-phase

method.[S2] The coupling reactions were performed by using 3.0 equiv. of Fmoc-protected amino acid, 3.0 equiv. of 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 3.0 equiv. of 1-hydroxybenzotriazole monohydrate (HOBt·H_2O) and 6.0 equiv. of *N*,*N*-Diisopropylethylamine (DIPEA) in *N*-methylpyrrolidone (NMP). Cleavage and side chain deprotection of peptides were carried out with 9.5 mL of TFA in the presence of 0.25 mL of triisopropylsilane and 0.25 mL distilled water by stirring for 1.0 h. After filtration, the reaction solution was concentrated under reduced pressure, and crude peptides were precipitated in cooled diethyl ether. Crude peptides were purified by RP-HPLC (column, COSMOSIL 5C18-ARII, ϕ10×250 mm). The HPLC solvents employed were ultrapure water containing 0.1% TFA (solvent A) and acetonitrile containing 0.1% TFA (solvent B). All peptides were obtained as TFA salts after lyophilization. Purified peptides were identified by ESI-MS. Pep1, *m/z* 838.9, calcd 839.0 $[M+2H]^{2+}$, *m/z* 559.6, calcd 559.6 $[M+3H]^{3+}$; Pep2 *m/z* 930.6, calcd 931.1 $[M+2H]^{2+}$, *m/z* 621.1, calcd 621.0 $[M+3H]^{3+}$; Pep3, *m/z* 852.4, calcd 852.5 $[M+2H]^{2+}$, *m/z* 568.6, calcd 568.7 $[M+3H]^{3+}$; Pep4, *m/z* 852.4, calcd 852.5 $[M+2H]^{2+}$, *m/z* 568.6, calcd 568.7 $[M+3H]^{3+}$; G-loop, *m/z* 690.6, calcd 690.8 $[M+2H]^{2+}$, *m/z* 460.8, calcd 460.8 $[M+3H]^{3+}$.

Expression of His-tagged hDHFR

The vector pColdII-hDHFR for expression of human dihydrofolate reductase (hDHFR) with His-tag at N-terminus was kindly gifted from Prof. Hiroshi Handa and Prof. Eiry Kobatake and transformed into *E. coli* BL21(DE3). The cells were grown in

100 mL LB medium containing 150 μg/mL ampicillin at 37ºC until an optical density at 600 nm (OD_{600}) increased to 0.5, then shaken at 15ºC for 1.0 h. Protein expression was induced at 15ºC for 24 h with 0.1 mM Isopropyl β-D-1-thiogalactopyranoside. The cells were harvested by centrifugation (4ºC, 9000 rpm, 20 min). The cells were re-suspended in Wash buffer (50 mM sodium phosphate, 150 mM NaCl, pH 7.0) and lysed by sonication (60 sec sonication, 10 cycles, Bioruptor, Cosmo Bio). Insoluble materials were removed by centrifugation (4ºC, 9000 rpm, 30 min) and supernatant was absorbed on 2 mL TARON resin equilibrated by Wash buffer in a plastic column for 20 min. The resin was washed with 10 mL Wash buffer (2 times). The resin-bound protein was eluted with 5 mL Elution buffer (50 mM sodium phosphate, 150 mM imidazole, 300 mM NaCl, pH 7.0). The fractions containing purified hDHFR were combined and dialyzed into excess PBS or 50% glycerol/MTEN buffer (50 mM MES, 25 mM Tris, 25 mM 2-aminoethanol, 100 mM NaCl, pH 7.0) (2 times). The concentration of purified hDHFR was determined based on UV absorption at 280 nm using the molar extinction coefficient of hDHFR (24750 M^{-1} cm^{-1}) in 6 M guanidine hydrochloride/20 mM sodium acetate buffer (pH 6.5).

Biotinylation of hDHFR

Biotin-AC_5-OSu was dissolved in DMSO to prepare 50 mM solution. hDHFR in PBS was biotinylated using 10 equiv. of biotin-AC_5-OSu. The mixture was rotated at 4ºC for 1.5 h and excess biotin-AC_5-OSu was removed by gel filtration chromatography (PD10 desalting column, GE Healthcare) using MTEN buffer.

Biopanning

Dynabeads M-280 Streptavidin (Streptavidin-magnetic beads) and NeutrAvidin-magnetic beads were used for biopanning. NeutrAvidin-magnetic beads were prepared using Dynabeads M-280 Tosylactivated according to the previous reports.[S3]

hDHFR-immobilized magnetic beads were prepared by mixing biotinylated hDHFR solution (2 μM, 50 μL in MTEN buffer) with Streptavidin- or NeutrAvidin-magnetic beads at 4ºC for 10 min. The magnetic beads were washed with MTEN buffer (500 μL, 3 times). After blocking of hDHFR-immobilized magnetic beads and phages (5×10^9 pfu) using 150 μL blocking buffer (MTEN buffer containing 1% (w/v) BSA or 1% (w/v) BSA/0.1% (v/v) tween20) for 30 min, respectively, hDHFR-immobilized magnetic beads and phages were incubated under several conditions shown in Table S1. The bound phage was eluted from the beads by incubating with 200 μL of 100 μM MTX in MTEN buffer for 15 min. The eluted phage was inoculated to *E. coli* XL1-Blue cells with helper phage (1×10^{11} pfu). The amplified phage was used for the next biopanning. After 9 round biopanning, phage clones were identified (Table S2).

Phage ELISA

A 96-well microplate (high binding, half area, Corning) was coated with 50 μg/mL NeutrAvidin in MTEN buffer (30 μL) at 4ºC for 2.0 h. The wells were washed 3 times with 100 μL MTEN buffer and blocked with 1% (w/v) BSA in MTEN buffer

(80 μL) at 4ºC for for 1.0 h, and washed 3 times with 120 μL MTEN buffer. Biotinylated hDHFR (2.0 μM, 50 μL) was added to the wells and incubated at 4ºC for 20 min. After wash with 100 μL MTEN buffer (3 times), each phage clone solution (2 nM, 50 μL) in MTEN buffer (containing 1% (w/v) BSA and 100 μM NADPH) or MTEN buffer (containing 1% (w/v) BSA, 100 μM NADPH and 10 μM MTX) was added into the wells, and incubated at room temperature for 1.0 h. The wells were washed 5 times with 100 μL MTEN buffer (containing 100 μM NADPH). Anti-phage antibody HRP conjugate in MTEN buffer was added to each well and incubated at room temperature for 1.0 h. The wells were washed 3 times with 100 μL MTEN buffer (containing 100 μM NADPH), and fluorogenic enzyme reaction was performed with QuantaBlu Fluorogenic Peroxidase Substrate Kit (Pierce). The fluorescence intensity was measured with microplate fluorometer (Twinkle LB 970, BERTHOLD TECHNOLOGIES GmbH & Co. KG) (Figure S1).

Secondary structural analyses of peptides by circular dichroism (CD) spectroscopy and attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy

Circular dichroism spectra were recorded on a J-720WI spectropolarimeter using a quartz cell with 0.1 cm path length at 25°C. All peptides were dissolved in 100 mM phosphate buffer solution containing 50% trifluoroethanol to prepare 100 μM solutions.

Infrared spectra were recorded on IRPrestige-21 with DuraSampl IR-II (Shimadzu). All peptides were dissolved in ultrapure water (500 μM). A 5.0 μL

aliquot of the solution was placed on a reflective surface of FT-IR instrument and air-dried. ATR-IR spectra were collected using refractive surfaces in air at room temperature. Number of scans was 100 times and Fourier transformed at a resolution of 4 cm^{-1}.

hDHFR inhibitory activity of peptides

Enzymatic activity of hDHFR was measured at 25°C on a Shimadzu UV-2550 spectrometer using a quarts cell with 1.0 cm path length. Initial rates of DHP reduction by hDHFR were determined by the absorption change of NADPH at 340 nm (ε = 6220 M^{-1} cm^{-1}) in MTEN buffer. To determine IC_{50} values of peptides, concentrations of hDHFR, DHP and NADPH were fixed as 15 nM, 1.0 μM and 100 μM, respectively, and concentrations of peptides were varied up to 200 μM. The inhibition constant (K_i) of Pep1 and Pep2 were estimated by kinetic analysis of hDHFR reaction in the absence and presence of these peptides. Kinetic parameters were estimated based on equation (1),[S4] because Pep1 and Pep2 worked as a competitive inhibitor (Figure S2).

$$\frac{1}{V_0} = \left(\frac{aK_m}{V_{max}}\right)\frac{1}{[S_0]} + \frac{1}{V_{max}} \quad a = 1 + \frac{[I]}{K_i} \quad (1)$$

Where V_0, V_{max}, K_m, K_i, $[S_0]$ and [I] mean initial reaction rate, maximal reaction rate, Michaelis constant, inhibition constant, substrate (DHP) concentration and inhibitor (Pep1 and Pep2) concentration, respectively. Concentrations of hDHFR and NADPH were fixed at 15 nM and 100 μM, respectively. Concentrations of Pep1 were 9.8 μM and 20 μM. Concentrations of Pep2 were 43 μM and 100 μM. DHP concentration was varied from 0.5 μM to 20 μM. An average value of 3 measurements was plotted for each point.

Binding analysis of peptides to hDHFR by isothermal titration calorimetry (ITC)

Isothermal titration calorimetry (ITC) was performed on a MicroCal VP-ITC calorimeter (GE Healthcare). The measurement was conducted at 30°C. Titrations were performed by injecting 20 or 25 aliquots of 10 μL of peptide/MTEN buffer solution (pep1: 180 μM, pep2: 195 μM) into the hDHFR/MTEN buffer solution (15 μM) every 5 min. The heat flow resulting from the binding of peptides to hDHFR was recorded as function of time and converted into enthalpies (ΔH) by integration of the appropriate reaction peaks (Figure S4). Dilution effects were corrected by subtracting the results of a blank experiment with MTEN buffer in place of peptide/MTEN buffer solution under identical experimental conditions. The binding parameters (K_d, ΔH, ΔS) were evaluated by applying one site model using software Origin (GE Healthcare).

Table S1. Biopanning conditions.

		Affinity condition		Wash condition	
round	hDHFR-beads	buffer	time (min)	buffer	times
1	SA[a]	BSA/MTEN[c]	30	MTEN[e]	3
2	NA[b]	BSA/MTEN[c]	30	MTEN[e]	5
3	SA[a]	BSA/MTEN[c]	30	MTEN[e]	8
4	NA[b]	BSA/MTEN[c]	30	MTEN[e]	8
5	NA[b]	BSA/MTEN[c]	30	MTEN/tween20[f]	8
6	SA[a]	BSA/ MTEN/tween20[d]	15	MTEN/tween20[f]	8
7	NA[b]	BSA/ MTEN/tween20[d]	15	MTEN/tween20[f]	8
8	SA[a]	BSA/ MTEN/tween20[d]	10	MTEN/tween20[f]	8
9	NA[b]	BSA/ MTEN/tween20[d]	10	MTEN/tween20[f]	8

a hDHFR-immobilized Streptavidin-magnetic beads

b hDHFR-immobilized NeutrAvidin-magnetic beads

c MTEN buffer containing 1% (w/v) BSA and 100 μM NADPH

d MTEN buffer containing 1% (w/v) BSA, 0.1% (v/v) tween20 and 100 μM NADPH

e MTEN buffer 100 μM NADPH

f MTEN buffer containing 0.1% (v/v) tween20 and 100 μM NADPH

Table S2. The amino acid sequences of identified phage clones.

Clone	Sequence of loop	Frequency
c1	PQEKV	21/40
c2	RWFEF	11/40
c3	LREPL	2/40
c4	LRLEP	4/40
c5	SLSDT	2/40

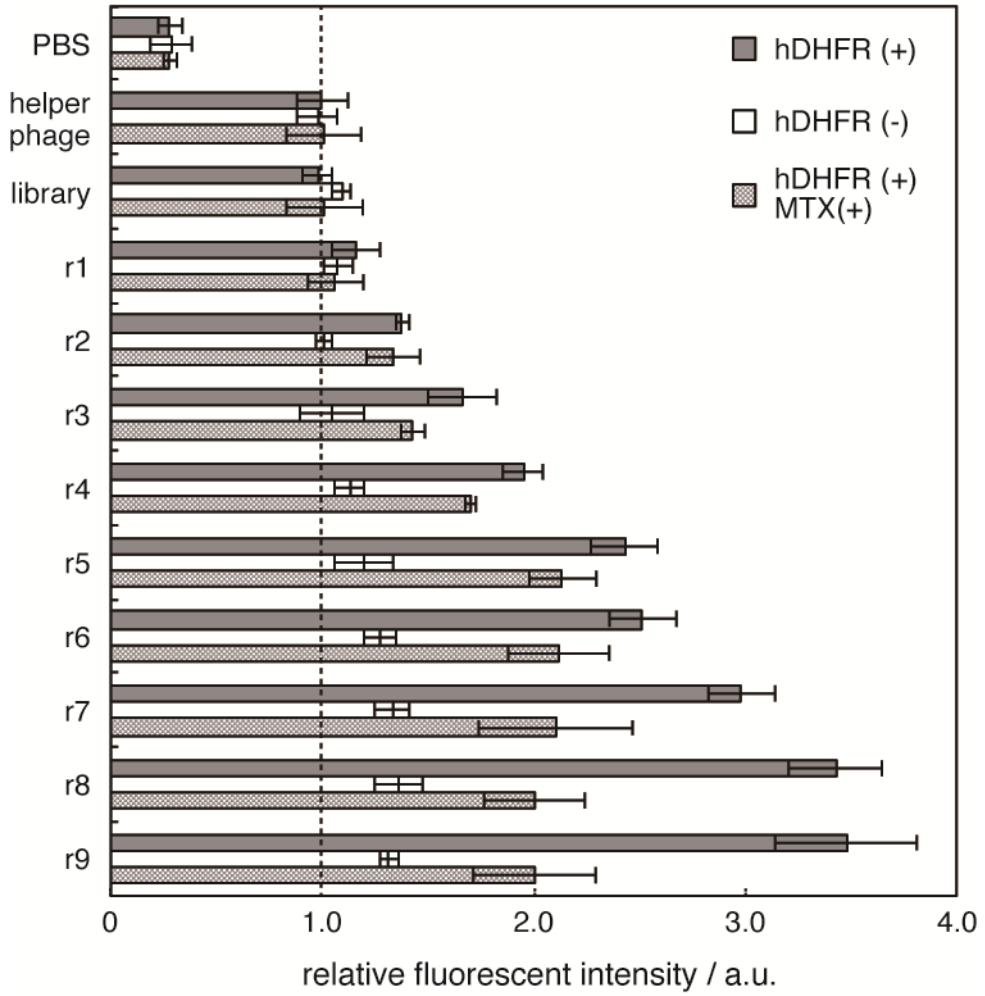


Figure S1. The phage ELISA experiment for the binding of phage pools to immobilized hDHFR. The fluorescent intensities due to the bound phages were detected in the absence (gray bar) and presence (checkered bar) of MTX (10 μM). For all samples, n = 3. Error bars represent the standard deviation.

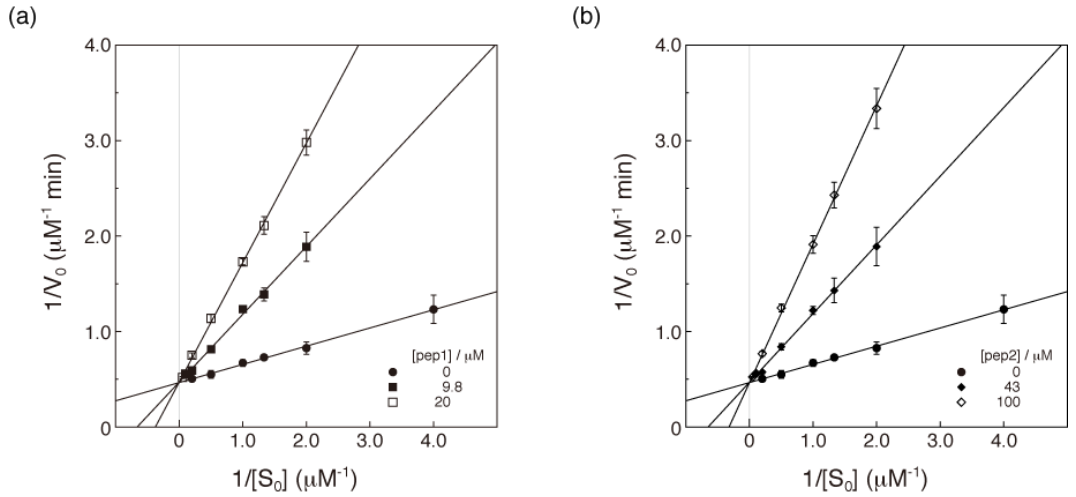
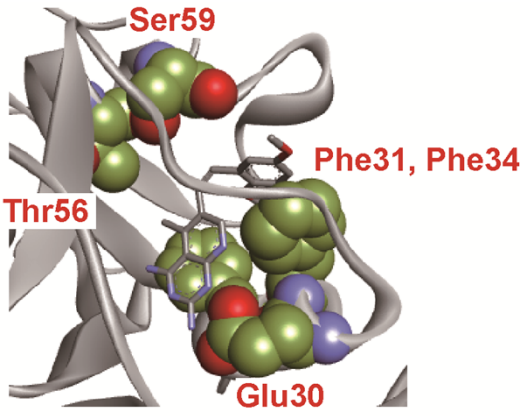
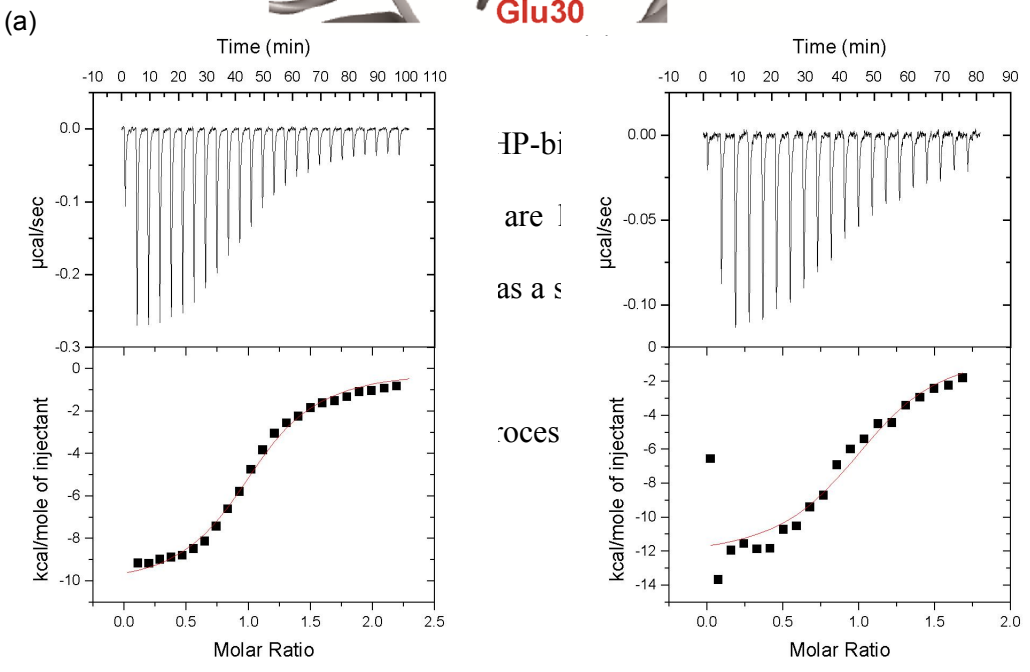


Figure S2. Kinetic analysis of hDHFR inhibitory activity by (a) Pep1 and (b) Pep2. Measurement conditions: [hDHFR] = 15 nM, [DHP] = 0.25-20 μM, [NADPH] = 100 μM, [Pep1] = 9.8 and 20 μM, [Pep2] = 43 and 100 μM at 25°C in MTEN buffer.





Measurement conditions: [hDHFR] = 15 μM, [pep1] = 180 μM (10 μL, 20 injections), [pep2] = 195 μM (10 μL, 25 injections), at 30°C in MTEN buffer (pH 7.0).

References

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