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## Dihydrofolate Reductase Inhibitory Peptides Screened from a Structured Design $\beta$ -Loop Peptide Library Displayed on Phage

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### Abstract

Enzyme inhibitory peptides with a loop structure stabilized by an antiparallel  $\beta$ -sheet scaffold ( $\beta$ -loop peptide) were obtained from a designed peptide phage library. Human dihydrofolate reductase (hDHFR) was used as the target enzyme. The obtained  $\beta$ -loop peptides were competitive inhibitors of hDHFR with micromolar inhibition constants and dissociation constants.

### Keywords

Peptide,  $\beta$ -sheet, Inhibitor, Phage display, dihydrofolate reductase

Enzyme inhibitors are important for drug discovery<sup>1</sup> because they influence various biological events and diseases, such as cancer,<sup>2</sup> viral infections<sup>3</sup> and Alzheimer's disease.<sup>4</sup> Peptide inhibitors against drug target enzymes are useful drug candidates. Various peptide inhibitors have been designed based on natural peptide substrates<sup>5</sup> and screened from various libraries.<sup>6</sup> Structurally constrained peptides are particularly promising because the side chains of the amino acids essential for inhibition can be appropriately oriented by using stable backbone structures as scaffolds.<sup>7-10</sup>

We previously designed and constructed a  $\beta$ -loop peptide phage library in which a loop structure composed of four or five randomized amino acids was stabilized by antiparallel  $\beta$ -strands (Figure 1). From the structured designed  $\beta$ -loop peptide library, insulin-binding peptides with selective and strong binding affinity were identified by an affinity-based screening.<sup>11</sup> A mannose-modified  $\beta$ -loop peptide phage library constructed by chemical modification of displayed peptides on phage provided peptides that selectively bind concanavalin A, a mannose-binding lectin.<sup>12</sup> In both cases, a stable  $\beta$ -loop structure was important for their strong binding affinity to targets because lacking antiparallel  $\beta$ -sheet structure decreased binding affinity. Based on these results, we screened our  $\beta$ -loop peptide phage library for peptides with enzyme inhibitory activity. Human dihydrofolate reductase (hDHFR), an enzyme associated with a folate metabolism, was selected as the target enzyme because DHFR is a drug target in cancer cells.<sup>13</sup> Small molecules such as methotrexate (MTX) can inhibit hDHFR, but hDHFR occasionally develops tolerance to MTX in cancer cells.<sup>14</sup> Peptide inhibitors frequently interact with target proteins with a large surface area; therefore, peptide inhibitors of hDHFR may also be effective against MTX-resistant

hDHFR. Our  $\beta$ -loop peptide scaffold is expected as a useful format to obtain inhibitory peptides against drug target enzymes.

A  $\beta$ -loop peptide phage library with a five-residue random sequence in the loop region was used for screening. Affinity-based screening of the  $\beta$ -loop peptides against hDHFR was performed using the competitive elution method with MTX. Biopanning was repeated 9 times with increasingly restrictive binding and washing conditions (Table S1) to obtain phages that displayed peptides binding hDHFR (Figure S1). Five different phage clones were then identified from the ninth-round phage pool by cloning and DNA sequencing of the individual clones. Two phage clones with PQEKV (c1) and RWFEF (c2) sequences in the loop region appeared with particularly high frequency (Table S2). Because these two phage clones exhibited greater binding to hDHFR in the phage ELISA experiment (Figure 2) than the other phage clones, c1 and c2 were selected as phage clones displaying stronger hDHFR-binding peptides.

To evaluate the secondary structure, inhibitory activity and binding affinity of the peptides screened by biopanning, the peptides were chemically synthesized. Four peptide sequences obtained by screening were synthesized as Pep1, Pep2, Pep3 and Pep4. A G-loop peptide with a GGGGG sequence in the loop was also synthesized as a control (Table 1).

The secondary structure of the peptides was analysed by circular dichroism (CD) spectroscopy and attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy (Figure 3). Pep1 exhibited negative broad shoulder peaks at around 216 nm and 205 nm, indicating that pep1 formed a loop-like structure composed of a  $\beta$ -sheet and  $\beta$ -turn. Pep2 exhibited the characteristic CD spectra of a  $\beta$ -sheet structure, with a negative maximum at 217 nm and a positive maximum at 197 nm.

Pep3 and Pep4 also displayed negative shoulder peaks at 220 nm and a negative maximum at 205 nm. In the IR spectra, Pep1-Pep4 displayed peaks around  $1625\text{ cm}^{-1}$  assigned to the  $\beta$ -sheet structure ( $1625\text{-}1635\text{ cm}^{-1}$ ).<sup>15</sup> In particular, Pep2 exhibited a maximum at  $1670\text{ cm}^{-1}$  and a shoulder peak at  $1695\text{ cm}^{-1}$  that were primarily assigned to a  $\beta$ -turn ( $1660\text{-}1700\text{ cm}^{-1}$ ) and antiparallel  $\beta$ -sheet ( $1685\text{-}1695\text{ cm}^{-1}$ ), respectively. These results suggest that all peptides obtained by screening formed  $\beta$ -loop structures consisting of a  $\beta$ -sheet and  $\beta$ -turn and that the most stable  $\beta$ -loop structure belonged to Pep2. By contrast, the G-loop exhibited little or no signal characteristic of a  $\beta$ -sheet structure in the CD and IR spectra.

The inhibition activities of the peptides against hDHFR were evaluated by kinetic analysis of the enzymatic activity of hDHFR (Supplementary section, Table 1). The 50% inhibitory concentrations ( $IC_{50}$ ) of Pep1 and Pep2 ( $9.8\text{ }\mu\text{M}$  and  $43\text{ }\mu\text{M}$ , respectively) were lower than those of Pep3 and Pep4 ( $IC_{50} > 200\text{ }\mu\text{M}$ ). The order of these peptides with respect to their  $IC_{50}$  values was well correlated with the binding amounts of the phage clones in the ELISA experiment. By contrast, the G-loop did not exhibit any inhibitory activity against hDHFR. Based on the results of the structural analyses, the inhibitory activity of Pep1 and Pep2, which have stable  $\beta$ -loop structures, was attributed to the loop region stabilized by the antiparallel  $\beta$ -sheet structure.

Kinetic analyses of hDHFR in the presence of inhibitory peptides revealed that both Pep1 and Pep2 are competitive inhibitors of hDHFR (Figure S2), with estimated inhibition constants ( $K_i$ ) of  $3.64\text{ }\mu\text{M}$  and  $15.4\text{ }\mu\text{M}$ , respectively (Table 1). These inhibition constants are consistent with the  $IC_{50}$  values of these peptides and indicate that Pep1 is a stronger inhibitor than Pep2. In hDHFR, Glu30, Phe31 and Phe34 are located near the substrate-binding site, and an electrostatic interaction between Glu30

and the Lys residue of Pep1 and a hydrophobic interaction between Phe31/Phe34 and the Val residue of Pep1 may be the driving forces behind Pep1 binding to hDHFR (Figure S3). Hydrogen bonding between Thr56/Ser59 of hDHFR and the Gln/Glu residues of Pep1 might assist the competitive binding of Pep1 to the substrate-binding pocket of hDHFR more effectively than Pep2. Although the inhibitory activities of Pep1 and Pep2 are weaker than that of MTX,<sup>14a</sup> inhibitory peptides with various inhibition modes are promising for the development of useful inhibitors against MTX-resistant hDHFR mutant proteins.

Finally, the binding affinity of Pep1 and Pep2 to hDHFR was evaluated based on the dissociation constant ( $K_d$ ) determined by isothermal titration calorimetry (ITC) (Table 1). The  $K_d$  value of Pep2 (12.0  $\mu\text{M}$ ) is similar to the  $K_i$  (15.4  $\mu\text{M}$ ), whereas, Pep1 exhibited a  $K_d$  value (1.37  $\mu\text{M}$ ) lower than its  $K_i$  (3.64  $\mu\text{M}$ ). These results indicate that Pep1 binds to the substrate-binding pocket of hDHFR more competitively than Pep2. In the thermodynamic parameters, Pep1 and Pep2 exhibited similar  $T\Delta S$  values of  $-0.28 \times 10^4$  J/mol and  $-0.24 \times 10^4$  J/mol, respectively, although the number of hydrophobic residues differs between Pep1 (one Val residue) and Pep2 (a Trp and two Phe residues) (Table 2). However, both Pep1 and Pep2 exhibited larger contribution of the  $\Delta H$  value ( $-3.40 \times 10^4$  J/mol and  $-2.85 \times 10^4$  J/mol, respectively), suggesting that the binding of both peptides to hDHFR is mainly enthalpy driven. In addition, the  $\Delta H$  value of Pep1 is more negative than that of Pep2, indicating that electrostatic interactions and/or hydrogen bonding play a larger role in the binding of Pep1 compared to Pep2. This is consistent with the inhibition model of Pep1 and Pep2 described above.

In conclusion, we have successfully obtained inhibitory peptides against hDHFR from a structured design  $\beta$ -loop peptide phage library. Secondary structural analyses revealed that all of the screened peptides maintained  $\beta$ -loop structures. Kinetic analyses revealed that Pep1 and Pep2, which both contain a stable  $\beta$ -loop structure, competitively inhibit hDHFR; however, Pep1 and Pep2 might inhibit hDHFR activity by binding in different manners to the substrate binding site. The binding of peptides to hDHFR is mainly enthalpy-driven, and electrostatic interactions and/or hydrogen bonding play a larger role in the peptide binding. Thus, the structured design  $\beta$ -loop peptide phage library was valuable in obtaining enzyme inhibitory peptides.

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### **References**

1. S. P. Gupta, *Chem. Rev.*, **1987**, *87*, 1183-1253.
2. (a) M. Lee, R. Fridman, S. Mobashery, *Chem. Soc. Rev.*, **2004**, *33*, 401-409. (b) A. V. Bieliauskas, M. K. H. Pflum, *Chem. Soc. Rev.*, **2008**, *37*, 1402-1413.
3. C. Reynolds, C. B. Koning, S. C. Pelly, W. A. L. Otterlo, M. L. Bode, *Chem. Soc. Rev.*, **2012**, *41*, 4657-4670.
4. A. K. Ghosh, H. L. Osswald, *Chem. Soc. Rev.*, **2014**, *43*, 6765-6813.
5. (a) H. M. Abdel-Rahman, T. Kimura, K. Hidaka, A. Kiso, A. Nezami, E. Freire, Y. Hayashi, Y. Kiso, *Biol. Chem.*, **2004**, *385*, 1035-1039. (b) X. Qiu, Z.-P. Liu, *Curr. Med. Chem.*, **2011**, *18*, 4513-4537.
6. (a) G. Castel, M. Chtéoui, B. Heyd, N. Tordo, *Molecules*, **2011**, *16*, 3499-3518. (b) K. Josephson, A. Ricardo, J. W. Szostak, *Drug Disc. Today*, **2014**, *19*, 388-399.
7. D. Fujiwara, Z. Ye, M. Gouda, K. Yokota, T. Tsumuraya, I. Fujii, *Bioorg. Med. Chem. Lett.*, **2010**, *20*, 1776-1778.
8. (a) C. Heinis, T. Rutherford, S. Freund, G. Winter, *Nat. Chem. Biol.*, **2009**, *5*, 502-507. (b) S. Chen, D. Bertoldo, A. Angelini, F. Pojer, C. Heinis, *Angew. Chem. Int. Ed.*, **2014**, *53*, 1602-1606. (c) C. Heinis, G. Winter, *Curr. Opin. Chem. Biol.*, **2015**, *26*, 89-98.
9. Y. Tokunaga, Y. Azetsu, K. Fukunaga, T. Hatanaka, Y. Ito, M. Taki, *Molecules*, **2014**, *19*, 2481-2496.
10. (a) Y. Yamagishi, I. Shoji, S. Miyagawa, T. Kawakami, Y. Goto, H. Suga, *Chem. Biol.*, **2011**, *18*, 1562-1570. (b) J. Morimoto, Y. Hayashi, H. Suga, *Angew. Chem. Int. Ed.*, **2012**, *51*, 3423-3427. (c) C. J. Hipolito, H. Suga, *Curr. Opin. Chem. Biol.*, **2012**, *16*, 196-203.
11. T. Sawada, K. Ishiguro, T. Takahashi, H. Mihara, *Mol. BioSyst.*, **2011**, *7*, 2558-2562.



12. K. Arai, H. Tsutsumi, H. Mihara, *Bioorg. Med. Chem. Lett.*, **2013**, *23*, 4940-4943.
13. B. I. Schweitzer, A. P. Dicker, J. R. Bertino, *FASEB J.*, **1990**, *4*, 2441-2452.
14. (a) S. K. Chunduru, V. Cody, J. R. Luft, W. Pangborn, J. R. Appleman, R. L. Blakley *J. Biol. Chem.*, **1994**, *269*, 9547-9555. (b) M. Lindgren, K. Rosenthal-Aizman, K. Saar, E. Eiríksdóttir, Y. Jiang, M. Sassian, P. Östlund, M. Hällbrink, Ü. Langel, *Biochem. Pharmacol.*, **2006**, *71*, 416-425.
15. (a) J. P. Moses, K. S. Satheeshkumar, J. Murali, D. Alli, R. Jayakumar, *Langmuir*, **2003**, *19*, 3413-3418. (b) S. Srisailam, H.-M. Wang, T. K. S. Kumar, D. Rajalingam, V. Sivaraja, H.-S. Sheu, Y.-C. Chang, *J. Mol. Biol.*, **2002**, *277*, 19027-19036.

Table 1. Peptide sequences, inhibitory activities and dissociation constants of peptides against hDHFR.

Peptide	Sequence	IC <sub>50</sub> / $\mu\text{M}^a$	$K_i$ / $\mu\text{M}^a$	$K_d$ / $\mu\text{M}^b$
Pep1	H-GKITVPQE <u>KVK</u> TYEG-NH <sub>2</sub>	9.8	3.64	1.37
Pep2	H-GKITVRWFE <u>F</u> KTYEG-NH <sub>2</sub>	43	15.4	12.0
Pep3	H-GKITVLR <u>REPL</u> KTYEG-NH <sub>2</sub>	> 200	- <sup>c</sup>	- <sup>c</sup>
Pep4	H-GKITVLR <u>L</u> EPKTYEG-NH <sub>2</sub>	> 200	- <sup>c</sup>	- <sup>c</sup>
G-loop	H-GKITVGGGGGKTYEG-NH <sub>2</sub>	no inhibition	no inhibition	- <sup>c</sup>

*a*, determined by the enzyme assay (see Supporting Information)

*b*, determined by the ITC assay (see Supporting Information)

*c*, not determined

Table 2. Thermodynamic parameters for the binding of peptides to hDHFR.

	$\Delta G$ ( $10^4$ J/mol)	$\Delta H$ ( $10^4$ J/mol)	$T\Delta S$ ( $10^4$ J/mol)	$K_d$ ( $\mu\text{M}$ )
Pep1	-3.40	-3.68	-0.28	1.37
Pep2	-2.85	-3.09	-0.24	12.0

The method details are described in Supporting Information.

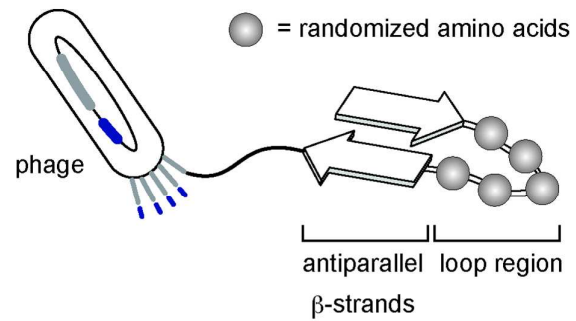
$\beta$ -loop peptide phage library

Figure 1. Illustration of  $\beta$ -loop peptide library displayed on phage.

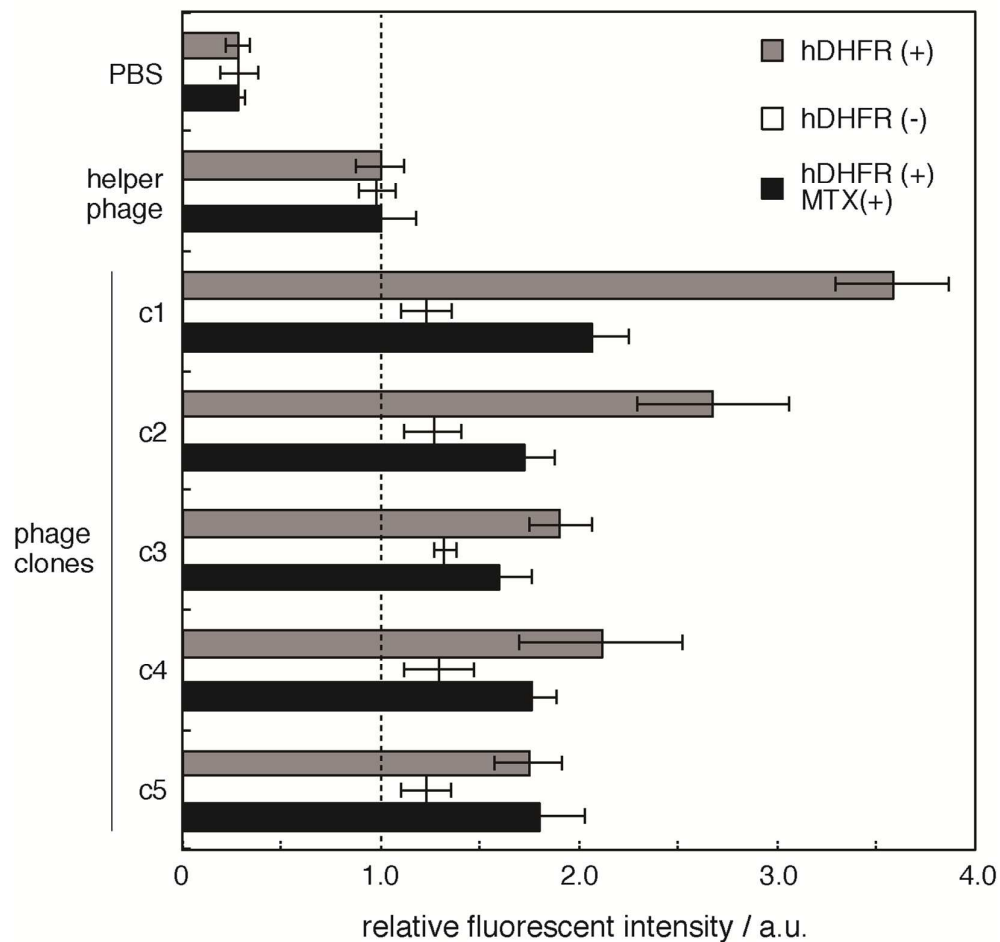


Figure 2. ELISA assays for the amounts of bound phage clones identified from the  $\beta$ -loop peptide phage library against hDHFR. The gray and white bars indicate the relative fluorescent intensity due to hDHFR-immobilized and non-immobilized microplates, respectively. The checked bar showed the relative fluorescent intensity due to hDHFR-immobilized microplates in the presence of MTX (10  $\mu$ M). hDHFR (2.0  $\mu$ M, 50  $\mu$ L) was used for the immobilization on NeutrAvidin-coated microplates and phage concentrations were 2.0 nM. For all samples,  $n = 3$ . Error bars represent the standard deviation.

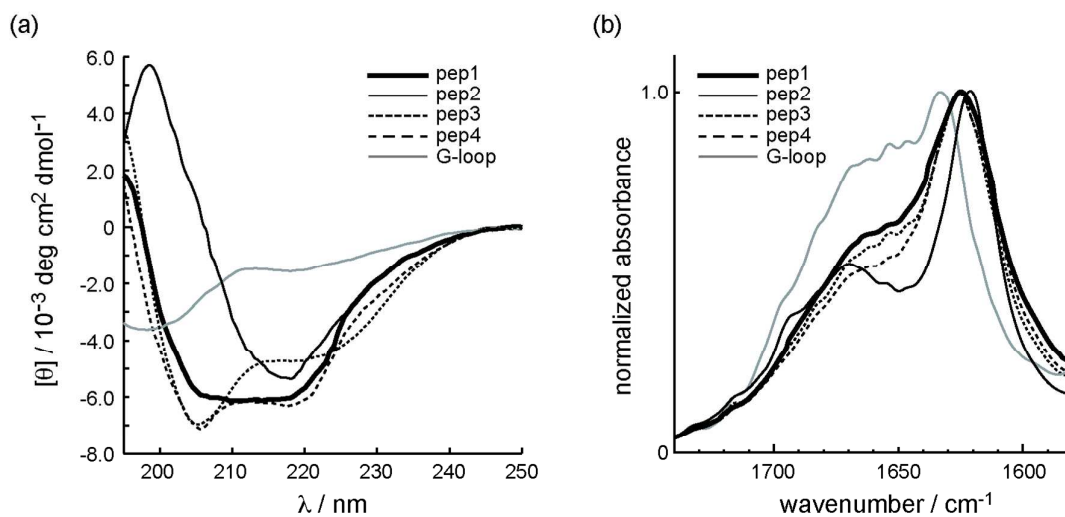


Figure 3. Secondary structural analyses of peptides by (a) CD spectra and (b) ATR-IR spectra measurements. All peptides were measured at 100  $\mu\text{M}$  in CD spectra measurement and at 500  $\mu\text{M}$  in IR spectra measurement. Pep1: bold solid line, pep2: solid line, pep3: dot line, pep4: dash line, G-loop: gray solid line.

Graphical abstract

