Anal. Chem. **2007,** *79,* 5006−5012

Immobilization of Malarial (*Plasmodium falciparum*) Dihydrofolate Reductase for the Selection of Tight-Binding Inhibitors from Combinatorial Library

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A simple procedure for selection of tight-binding inhibitors of mutant dihydrofolate reductases from *Plasmodium falciparum* (PfDHFRs) based on preferential binding to the enzyme immobilized on a Sepharose column has been described. PfDHFRs with a cysteine residue at the C-terminal have been prepared in order to immobilize to a thiopropyl-Sepharose gel via S−S linkage. The amount of immobilized DHFRs was estimated to be 4−5 mg/g of dried gel, and the activities of bound DHFRs were comparable to that of free enzymes. The prepared immobilized enzyme has been used for the selection of tight-binding inhibitors from combinatorial libraries, based on the affinities of each ligand with the enzyme. Free ligands were then identified and analyzed quantitatively by high-performance liquid chromatography−mass spectrometry, and the components with high binding affinity of the library could thus be realized. Results could be confirmed by quantitative analysis of the bound ligands released from the enzyme by guanidine hydrochloride treatment.

Plasmodium falciparum dihydrofolate reductase-thymidylate synthase (PfDHFR-TS) is a validated target of antifolate antimalarial drugs[1−3] such as pyrimethamine (Pry) and cycloguanil (Cyc), which are commonly used clinically for the treatment of malaria infection. Since the emergence of the resistant strain of malaria parasites to these inhibitors became widespread, there has been an urgent need to search for new drugs to combat the resistant malaria. The molecular mechanism for resistance to PfDHFR inhibitors has been shown to be due to point mutation at various sites of the parasite gene sequence, leading to the decrease in binding affinity of inhibitor to the enzyme. Mutation of one or more residues at amino acid positions 16, 51, 59, 108, and 164 of PfDHFR were identified to be involved in antifolate resistance.[4−9] Based on modeling of wild type and mutant PfDHFRs, a number of inhibitors were synthesized and screened against the enzymes individually and found to be effective against both wild type and some mutant PfDHFRs.[10−13]

The advent of combinatorial chemistry techniques offers the means for rapid generation of a large number of structurally related compounds. However, identification of potential leads from the combinatorial libraries required an effective selection system that can single out the tight-binding inhibitors for further characterization and development. Recently, Kamchonwongpaisan et al. have developed an ultrafiltration method for the selection, based on the dissociation constant of the enzyme with each inhibitor.[14] In the stoichiometric selection, the limitation of this technique is its dependence on the solubility of the target enzyme, which affected the compound concentration in the libraries: i.e., the larger the size of the library, the lower the concentration of each inhibitor.[14]

It has been reported that immobilized enzymes are useful tools in many areas such as for enzyme sensors, enzyme reactors, or enzyme catalysis in organic synthesis.[15−19] Dihydrofolate reductase enzymes were also immobilized onto the solid support in different

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10.1021/ac070215s CCC: $37.00
Published on Web 05/26/2007

manners and purposes.[15,16] In this paper, we report the method for immobilization of PfDHFR to the Sepharose gel for use in the selection of tight-binding inhibitors from combinatorial libraries. By using the immobilized enzyme, the unbound and released bound compounds can easily be separated from the reaction mixture for determination and have no limitation on the solubility of the enzyme.

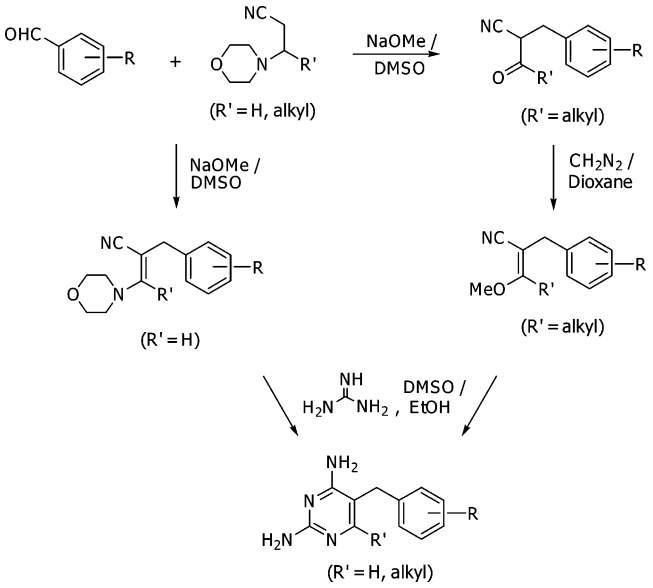
EXPERIMENTAL SECTION

Materials and Methods. For the synthesis of trimethoprim analogues, reagents were purchased from Fluka, Merck, and Sigma-Aldrich Ltd. and liquids were distilled before use. For enzyme studies, chemicals were obtained from Sigma-Aldrich Ltd., Merck, and BDH and were used without further purification. Oligonucleotides were made to order from Bioservice unit at our research center. Thiopropyl Sepharose 6B was purchased from Amersham Biosciences. Nuclear magnetic resonance spectra were recorded in DMSO-d_6 on a Bruker AV 500D spectrometer; chemical shifts are reported in parts per million (ppm). Mass spectra were recorded on a Micromass LCT using an electrospray ionization technique.

Enzyme Preparation. Double mutant PfDHFR[20] and PfDHFR-TS[21] (C59R+S108N) were cloned and expressed in *Escherichia coli* BL21(DE3) pLysS. Two modified PfDHFR enzymes with an eight-amino acid spacer and a Cys at the carboxylic terminal were engineered. The spacers of the enzyme were designed based on its natural amino acid sequences (KMLNEQNC, named K1NCR) and modified sequences (KMLNGGGC, named K1NGCR) at the junction region between DHFR and TS domains. These genes were PCR amplified from the available plasmid carrying the natural pfdhfr-ts gene[21] using 5′-GCC AGC **AAG CTT** ATG ATG GAA CAA GTC TGC GAC GTT-3′ as the forward primer and 5′-CTC CGC **GGT ACC** TTA ACA ATT TTG TTC ATT TAA CAT TTT A-3′ or 5′-CTC CGC **GGT ACC** TTA ACA CCC TCC GCC ACC TAA CAT TTT ATT ATT CGT TTT CTT-3′ as the reverse primer for K1NCR and K1NGCR, respectively. The PCR protocol was initiated with 95 °C for 5 min, followed by 30 thermal cycles (1-min denaturation at 95 °C, 1-min annealing period at 50 °C and 2-min extension period at 72 °C) and 10-min final extension at 72 °C. The PCR products of 732 base pairs were purified, restriction enzyme cut, and ligated back into the same modified pET17b. The DNA sequences of the constructs were verified by DNA sequencing. Protein expression and purification by Methotrexate-Sepharose affinity column were carried out as previously described.[20]

Immobilization of Enzyme to Thiopropyl-Sepharose 6B. For large-scale preparation, dried thiopropyl-Sepharose 6B gel (1 g) was suspended in 20 mL of buffer A (20 mM phosphate buffer, pH 7, 0.1 mM EDTA, 50 mM KCl, and 20% glycerol). The mixture was air-aspirated to remove oxygen and bubbled with nitrogen through the solution. Enzyme (20 mg) was added to the gel suspension and mixed gently at 4 °C overnight. The gel−enzyme suspension was transferred to a column and the unbound enzyme was removed by the continuous flow of 100 mL of buffer. The washed enzyme-bound gel was then resuspended in the same buffer and stored at 4 °C until used.

Scheme 1. Synthetic Route for Trimethoprim Derivatives



Determination of the Amount of Enzyme Bound to the Thiopropyl-Sepharose Gel. Fifteen portions of 10 mg of dried thiopropyl-Sepharose 6B gel were suspended in 200 μL of oxygen-removed buffer A. Each gel suspension was combined with 200 μL of the enzyme solution containing different amount of DHFR enzyme (5−600 μg). The mixtures were mixed gently at 4 °C. After 5 h of incubation, the suspensions were centrifuged for 10 min at 12 000 rpm at 4 °C, and the supernatant from each tube was collected for the enzyme assay. The enzyme activity in the supernatant solution was plotted against the amount of enzyme added to the gel suspension.

Enzyme Assay. The activity of PfDHFRs was determined spectrophotometrically at 25 °C according to the method previously described.[20] The reaction (1 mL) contained 10× DHFR buffer (50 mM *N*-[tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid], pH 7.0, 75 mM β-mercaptoethanol, 1 mg/mL bovine serum albumin), 100 μM each of the substrate dihydrofolate and cofactor NADPH, and appropriate amount of enzyme solution. In the case of immobilized enzyme, there is no β-mercaptoethanol in the reaction mixture and the gel-bound enzyme was placed instead of the enzyme solution. Thiopropyl-Sepharose gel was added to the reaction mixture for blank measurement.

Construction of the Collections of Ligands. Pry and Cyc were purchased from Sigma-Aldrich Ltd. Trimethoprim (Tmp) and their derivatives were synthesized (Scheme 1) individually according to the method described previously,[11,12,22] and their K_i values were measured by the conventional kinetic experiment.[23] Two collections of ligands were prepared for use in the stoichiometric selection. The first collection was a mixture of an equimolar amount of pyrimethamine, cycloguanil, and trimethoprim, which

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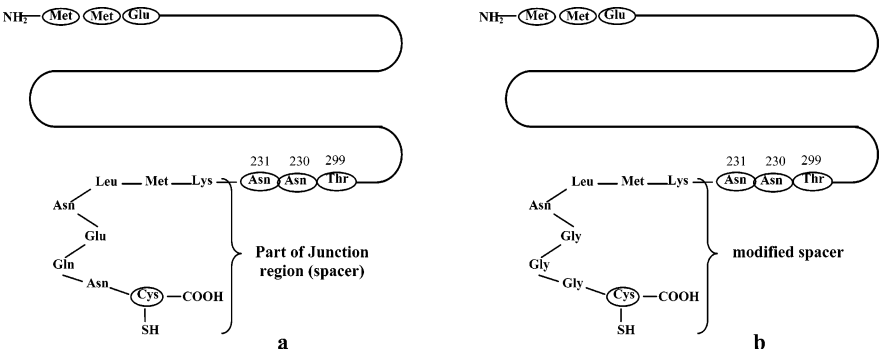

Figure 1. Schematic representation of extended DHFRs, which were constructed in this study: (a) DHFR-KMLNEQNC (K1NCR) and (b) DHFR-KMLNGGGC (K1NGCR).

was used for technique validation. The second collection was a mixture of an equimolar amount of trimethoprim and its 17 analogues, which was constructed on the basis of the K_i values of each compound against the double mutant enzyme (C59R+S108N). The stock solution of each library was prepared in DMSO.

Selection of Tight-Binding Inhibitors. One milliliter of the collection containing 270 μM ligands and 100 μM NADPH in buffer A was added into the column of PfDHFR immobilized gel (1 g of dried gel, contained 0.18 μmol of K1NCR) for the test and into the column of Sepharose gel for control. After 5-min incubation at 25 °C, unbound ligands were eluted from the column and collected for further analysis using HPLC. The column was briefly washed with buffer and treated with 1 mL of 6 M guanidine hydrochloride containing 12% DMSO to release bound ligands from the enzyme. The release compounds were then eluted from the column for further analysis by HPLC. The peak area of each ligand was determined.

HPLC and LC−MS Analyses. HPLC analyses were performed on a Waters 600 system equipped with a Waters 996 photodiode array detector, using a reversed-phase column (Atlantis C18, 5 μm, 4.6 × 250 mm) and acetonitrile and 25 mM ammonium acetate (pH 4.5) as the mobile phase at a flow rate of 1 mL/min. The separation was started with 15% acetonitrile, followed by stepwise gradient from 15 to 25, 25 to 35, and 35 to 70% at 5−10, 25−60, and 70−80 min, respectively. Then the separation continued at 70% actonitrile until the last compound was collected. The chromatograms were detected through absorption at 254 nm for peak area determination. Compound identification was achieved by LC−MS (Micromass) using the same column and solvent system. In the case of compounds with similar mass, standards of individual compounds were used to locate their peaks by HPLC under the same separation protocol.

RESULTS AND DISCUSSION

Design and Expression of Mutant DHFRs. The SH group of the cysteine (Cys) residue is very useful for immobilization of an enzyme to the solid support[15,24] because of its easy bond formation under mild conditions. For example, modified *E. coli* DHFR with a Cys residue at the C-terminal has been successfully immobilized to a thiopropyl-Sepharose gel.[15] In this study, PfDHFRs were designed to immobilize to thiopropyl-Sepharose via an S−S bond. PfDHFRs with a Cys residue at the C-terminal were constructed with the extension of some amino acids as a spacer to provide the flexibility for the enzyme. Since the DHFR domain of the bifunctional enzyme PfDHFR-TS joined to the TS domain by the junction region (JR) of which the amino acid residue at position 8 is Cys, double mutant (C59R+S108N) enzyme extended with eight amino acids of the JR was constructed and named K1NCR (Figure 1a). Thus, the eight amino acid residues of the JR acted as a spacer. Alternatively, it is possible that bond formation of the enzyme to the solid support might be restricted due to the steric effect of the large side chains of Glu, Gln, and Asn attached to Cys; therefore, three glycine (Gly) residues were introduced in place of these amino acid in the modified spacer and the engineered enzyme was named K1NGCR (Figure 1b).

Following enzyme purification, dithiothreitol (DTT) was removed from the enzyme solution by gel filtration through a small column of Sephadex G-50 prior to immobilization. This is to prevent the cleavage of S−S bond between the enzyme and the solid support in the immobilization process and to keep the S−S bond intact throughout the study. The specific activities of free K1NCR and K1NGCR enzymes after DTT removal were 71.8 and 80.3 units/mg of protein, respectively, which were similar to that of the parent C59R+S108N double mutant enzyme, K1 (90.1 units/mg of protein). The result indicated that the extended amino acids and the removal of DTT did not affect the enzyme activity.

Immobilization of DHFRs on Sepharose Gel. The amount of enzyme bound to thiopropyl-Sepharose gel was determined by a titration-based experiment.[15] Following incubation of 10 mg of the gel with various amounts of PfDHFR enzymes ranging from 5 to 600 μg, unbound enzyme was separated and the activity of enzyme was determined. Figure 2 shows the relationship between the total enzyme added to the gel suspension and the activity of unbound enzyme detected in supernatant solution. At low amounts of enzyme added, 5−40 μg, the activity of DHFR enzyme was undetectable to very low. With the increasing amount of enzyme, the enzyme activity of the unbound fraction became detectable. This indicated that, after saturation of the immobilization of enzyme onto the Sepharose gel, further addition of enzyme merely increased the amount of unbound enzyme, and therefore, the enzyme activity in the supernatant solution showed a linear relationship with the amount of added enzyme. The total amount of enzyme bound to the gel can then be estimated by extrapolating the linear line back to zero (x-axis intercept). This estimation is based on the assumption that the disappearance of enzyme activity

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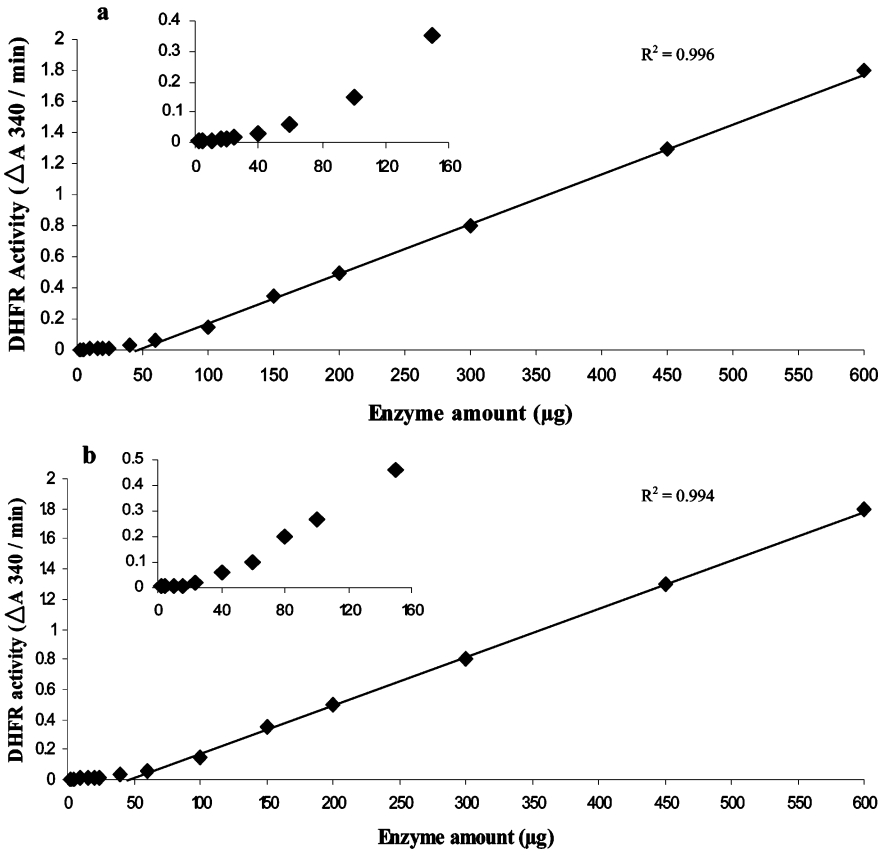
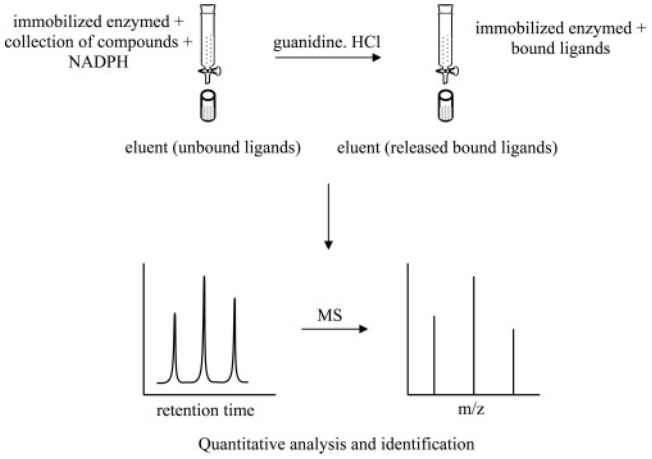


Figure 2. Immobilization of enzyme K1NCR (a) and K1NGCR (b) on thiopropyl-Sepharose 6B gel.

Scheme 2. General Protocol for Stoichiometric Selection



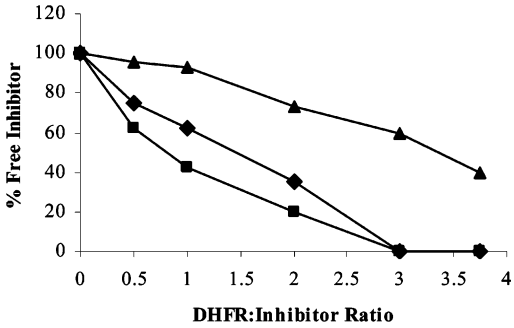


Figure 3. Selective binding of a collection containing pyrimethamine (◆), cycloguanil (■), and trimethoprim (▲) by an immobilized DHFR. The total concentration of ligands was fixed at 50 μM while the concentration of the enzyme was varied from 0, 25, 50, 100, 150, to 188 μM to give the molar ratio of enzyme to total ligands at 0, 0.5, 1.0, 2.0, 3.0, and 3.76, respectively.

from supernatant solution is immobilized onto the gel support; thus, the maximum estimate could be obtained from this method. The calculated specific activity of the enzyme would decrease as the amount of immobilized enzyme estimated increased. Therefore, it was not unreasonable to assume that this method gave the minimum specific enzyme activity. It was estimated that the amount of bound K1NCR and K1NGCR enzymes on the gels were 5 and 4 mg/1 g of dried gel as shown in Figure 2a and b, respectively. The calculated specific activities of the immobilized enzymes were 27.3 and 21.5 units/mg of protein for K1NCR and K1NGCR enzymes, respectively (using a maximum estimate value of each enzyme). A more accurate figure was also obtained by determining the amount of enzyme by protein determination[25] following DTT treatment to release the enzymes from the gel. By this direct determination, the amounts of bound K1NCR and K1NGCR enzymes on the gel were 4.4 and 3.2 mg/g of dried gel, respectively, which were similar to the values determined by the titration method. Upon release from the gel, the specific activities of the free enzymes (40.7 and 38.3 units/mg of protein) were comparable to those of the immobilized enzymes.

Technique Validation. The general procedure for the stoichiometric selection of tight-binding inhibitors by an immobilized enzyme is outlined in Scheme 2. Ligands with high affinity should bind tightly to the enzyme and the unbound ligands could then be eluted from the column for further quantitative analysis and

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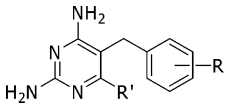
Table 1. Structure, Retention Time, Mass, and K_i Value against Double Mutant PfDHFR of Each Ligand in the Collection



Cpd	peak	R	R′	retention time	K_i double mutant (nM) expt	K_i double mutant (nM) calcd	molecular mass calcd	molecular mass $M + H^+$ (m/z)
T1	1	4-$N(CH_3)_2$	H	12.1 ± 0.3	449.8 ± 20.1	410.65	243.31	244.15
Tmp	2	3,4,5-$(OMe)_3$	H	13.6 ± 0.5	242.1 ± 40.1[a]	-	290.32	291.14
T2	3	3,4,5-$(OMe)_3$	Me	16.8 ± 0.1	520.3 ± 122.8[a]	639.42	304.35	305.16
T3	4	2,4-$(OMe)_2$	H	20.4 ± 0.1	762.2 ± 37.4	890.06	260.29	261.14
T4	5	4-OEt	H	21.4 ± 0.1	199.8 ± 20.3	238.12	244.30	245.14
T5	6	4-Br	H	24.7 ± 0.1	285.8 ± 47.0	219.97	278.01	279.02
T6	7	2,3-(C_4H_4)-	H	28.2 ± 0.1	222.1 ± 80.2	169.05	250.30	251.13
T7	8	4-Pr^i	H	42.2 ± 0.4	496.5 ± 54.1	506.48	242.32	243.16
T8	9	4-OCH_2-[3,4,5-$(OMe)_3$]Ph	H	43.3 ± 0.1	5.2 ± 0.3[a]	13.21	396.45	397.19
T9	10	3-OEt-4- OCH_2-[3,4,5-$(OMe)_3$]Ph	H	46.8 ± 0.1	2.2 ± 0.1[a]	12.49	440.50	441.21
T10	11	3-OBu^n	H	50.9 ± 0.1	288.6 ± 29.2	310.47	272.35	273.17
T11	12	4- Bu^t	H	52.8 ± 0.2	134.5 ± 18.5	113.87	256.35	257.17
T12	13	3-OPh	Me	54.8 ± 0.1	15.1 ± 1.8	22.16	306.37	307.16
T13	14	3-OEt-4-OCH_2Ph	H	59.0 ± 0.1	9.7 ± 1.4[a]	15.82	350.42	351.18
T14	15	3-OMe-4-OCH_2Ph	Et	60.3 ± 0.1	60.1 ± 8.1[a]	74.33	364.45	365.20
T15	16	4-$OC_3H_6OCH_2$Ph	H	68.1 ± 0.1	6.7 ± 0.5	15.29	364.45	365.20
T16	17	3- OCH_2Ph-4-OCH_2Ph	Me	82.9 ± 0.1	80.0 ± 10.3[a]	98.98	426.52	427.21
T17	18	4-OC_3H_6O-[2,4,5-$(Cl)_3$]Ph	H	87.1 ± 0.1	39.1 ± 3.1	35.46	452.05	453.07

[a] Data from ref 22.

identification by HPLC and LC−MS. K_i values of each ligand in the library could be calculated individually using eq 1[14] by mixing the reference compound with known K_i value to the library as an internal standard.

$$K_i \text{ value of ligand } i, \quad Ki_i = \frac{[l_i]\ [El_{ref}]}{[El_i]\ [l_{ref}]} \cdot Ki_{ref} \qquad (1)$$

$[l_i]$, $[El_i]$, $[l_{ref}]$, and $[El_{ref}]$ are concentrations of free ligand i, enzyme−ligand complex, reference, and enzyme−reference complex, respectively, where

$$[l_i] = \frac{\text{peak area of } l_i \text{ in the test}}{\text{peak area of } l_i \text{ in control}} \cdot l_{ti}$$

$$[El_i] = l_{ti} - [l_i]$$

$$l_{ti} = \text{the total concentration of ligand } i$$

$$[l_{ref}] = \frac{\text{peak area of } l_{ref} \text{ in the test}}{\text{peak area of } l_{ref} \text{ in control}} \cdot l_{tref}$$

$$[El_{ref}] = l_{tref} - [l_{ref}]$$

$$l_{tref} = \text{the total concentration of reference}$$

Since the activities of two immobilized enzymes were comparable, nonmodified enzyme K1NCR was selected for further study. The theoretical expectation of the binding of immobilized enzyme with the mixture of inhibitors was tested by titration of the enzyme with a mixture of equimolar of each Pyr, Cyc, and Tmp.[14] Then the amount of free inhibitors was determined. Figure 3 shows that the concentrations of free ligands decrease sequentially in the order of Cyc, Pyr, and Tmp as expected. Using eq 1 and data in Figure 3 at the enzyme/ligand ratio of 2, where all ligands share the binding to the enzyme at less than 100% binding, Pyr as the reference with K_i value of 53.9 nM, the calculated K_i values of Cyc and Tmp were 33.3 and 270.9 nM, respectively. These values are comparable with the previously reported K_i values of 42.63 ± 6.3 and 242.1 ± 40.1 nM, respectively. This demonstrated that the principle of stoichiometric selection of ligands was applicable to an immobilized PfDHFR and their ligands and the accurate K_i value of each compound in the library could be calculated by this method.

Construction and Identification of Trimethroprim Derivatives in the Collection. A series of Tmp derivatives has been specifically developed against plasmodial DHFR mutants with S108N mutation based on its flexible side chain on the pyrimidine ring that can avoid steric clash with the S108N.[22] Tmp and some derivatives were verified experimentally as competitive binding inhibitors against the double mutant enzyme (C59R+S108N) using the classical double-reciprocal plots (data not shown). The K_i values of these[22] and other DHFR inhibitors[10−13,20] were therefore calculated based on eq 2[26] or using nonlinear least-squares fit of the data to the Michaelis−Menten equation.[23]

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_m}} \qquad (2)$$

where IC_{50} is the concentration of the inhibitor that inhibits catalytic activity of the enzyme by 50%.

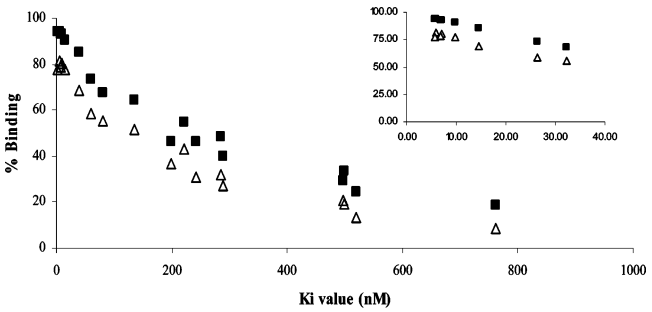


Figure 4. Correlation of percentage of binding and K_i value of ligands upon selection by immobilized enzyme using an excess molar ratio of enzyme to total ligands of 1.0:1.5; percent binding from bound ligands (△) and percent binding from unbound ligands (■).

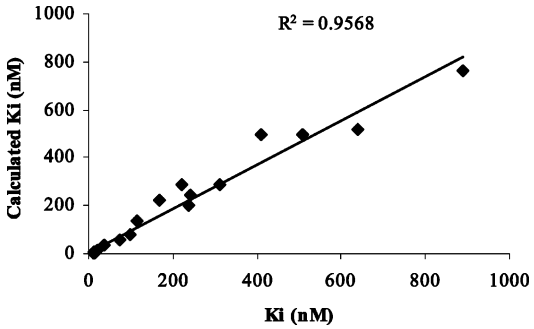


Figure 5. Correlation between K_i values of ligands in the collection from calculated and measured kinetically.

If a tight binding inhibitor was obtained, as defined by an inhibitor whose IC_{50} value observed is similar to the concentration of total enzyme in the sample, the calculation of the K_i value was based on eq 3, where total concentration of the enzyme [E] was also taken into account.[26]

$$K_i = \frac{IC_{50} - \frac{[E]}{2}}{1 + \frac{[S]}{K_m}} \quad (3)$$

The collection of Tmp and its derivatives was constructed as a representative of the combinatorial library. Successful evaluation of the collection would clearly imply that this method can be used for the screening of a library of potential inhibitors from combinatorial synthesis. In order to generate the collection of the compounds with a variety of binding affinities, a collection of 17 trimethoprim derivatives together with trimethoprim itself as reference was constructed based on their K_i values against the double mutant enzyme (C59R+S108N), which was measured by a conventional kinetic method.[23] This 18-compound-collection was subjected to HPLC analysis using a reversed-phase column and stepwise gradient of 15−70% acetonitrile and 25 nM ammonium acetate (pH 4.5), and each compound was identified by LC−MS. The amount of each compound was determined from the peak area at 254 nM. The structures, K_i values, and analysis results of these ligands in the library are summarized in Table 1.

Selection of Lead Compounds from the Collection by the Immobilized Enzyme. The binding assay was carried out with an excess molar ratio of 1.5/1.0 ligands to the enzyme, i.e., 270 μM total ligands (15 μM each) to 180 μM enzyme. The unbound ligands were first eluted from the column and then analyzed by HPLC. The missing compounds from the chromatogram, compared to the control, were determined as bound ligands. These were confirmed by the analysis of bound ligands, which were later released from the enzyme by guanidine hydrochloride. Ligands with lower K_i values (higher affinities) competed for the enzyme much more readily and bound to the enzyme at higher extent than those with higher K_i values, as shown by a linear correlation between percent binding to the enzyme from both unbound and bound ligands (calculated from eqs 4 and 5, respectively) and K_i values in Figure 4. It is worth mentioning that percent binding determined from bound ligands were slightly lower than those from unbound ligands, which is presumably due to the dissociation of weak binding complexes (high K_{off} rate) in the washing step.[14] Using trimethoprim as a reference with a K_i value of 242.1 applied to eq 1, the calculated K_i value of each derivative obtained from the data of unbound fraction was comparable to that measured kinetically (Table 1) as shown by the linear relationship between K_i values from both methods in Figure 5.

$$\% \text{ binding (from unbound)} = 100\left(1 - \frac{\text{peak area of the test}}{\text{peak area of control}}\right) \quad (4)$$

$$\% \text{ binding (from bound)} = 100\left(\frac{\text{peak area of the test}}{\text{peak area of control}}\right) \quad (5)$$

The results indicated that this method could efficiently be used for the selection of tight-binding inhibitors from the collection, and the K_i value of each ligand could be predicted reasonably accurately. It is unfortunate that reuse of the immobilized enzyme was not possible due to the denaturation of the enzyme by guanidine hydrocholoride. However, the advantages of this method over the free enzyme method[11] are that a higher amount of enzyme can be used, leading to a possible screening of a larger size of collection with higher concentration of ligands and thus increase in the sensitivity and accuracy of detection by quantitative HPLC analysis. Thus, this system should be a useful tool for a primary screening or large-scale selection of tight-binding inhibitors from combinatorial syntheses or libraries of natural products.

CONCLUSION

We have shown that PfDHFRs could be immobilized onto Sepharose gel via an S−S bond with the estimated amount of 4−5 mg/g of dried gel and could be used for the selection of tight-binding inhibitors from combinatorial libraries. By this method, both bound and unbound inhibitors were easily separated from the reaction mixture for further quantitative analysis and identification by HPLC and LC−MS. Since there was no limitation on the solubility of the immobilized enzyme, a higher concentration of the enzyme should be applicable. Thus, the higher concentration of each ligand could be used and a more accurate detection can be obtained from quantitative HPLC analysis.

ACKNOWLEDGMENT

TRF grant for new researcher from the Thailand Research Fund to C.T. and supports from Medicines for Malaria Venture

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(MMV) and Wellcome Trust to Y.Y. are gratefully acknowledged. S.K. is a HHMI international research scholar.

SUPPORTING INFORMATION AVAILABLE

Listing of spectroscopic data of Tmp derivatives, HPLC spectrum of the collection of ligands, and Lineweaver−Burk plots of the PfDHFR-C59RS108N by Tmp and derivative. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review February 1, 2007. Accepted April 22, 2007.

AC070215S