A simple method for screening combinatorial and other libraries of inhibitors of malarial (Plasmodium falciparum) dihydrofolate reductase (PfDHFR) has been developed, based on the affinities of the inhibitors with the enzyme. In the presence of limiting amounts of the enzyme, a number of inhibitors in the library were bound to extents reflecting the relative binding affinities. Following ultrafiltration and guanidine hydrochloride treatment to release bound inhibitors, the amounts of free and bound inhibitors could be determined by high-performance liquid chromatography and liquid chromatography–mass spectrometry. The differences in the patterns reflected the binding of high-affinity components compared with the other members in the library. A good correlation was found between the inhibition constants ($K_i$ values) and the extent of binding of inhibitors to wild-type, double (C59R+S108N) and quadruple mutant (N51I+C59R+S108N+I164L) of PfDHFR, as well as human DHFR. In addition to identifying lead components of the libraries with high affinities (low $K_i$ values) and stabilities (low $k_{off}$ rates), this simple method also provides an alternative way for quickly and accurately calculating enzyme binding affinities of inhibitors in combinatorial chemical libraries.

There are presently very few effective drugs against malaria, although it continues to be a major health burden of endemic developing countries, responsible for some 500 million cases and over 1 million deaths per year globally.1,2 Furthermore, development of resistance against these drugs poses urgent problems, the solutions to which involve finding new drugs effective against the resistant parasites. Development of new anti-folates against parasites resistant to such drugs as pyrimethamine (Pyr) and cycloguanil (Cyc) should help in solving these problems. Parasite dihydrofolate reductase (DHFR)-thymidylate synthase is the validated target of anti-folate antimalariais,3,4 and it has been shown that resistance of Plasmodium falciparum (Pf) to such drugs as Pyr and Cyc resulted from point mutations in the PfDHFR, notably at residues 16, 51, 59, 108, and 164, the accumulation of which led to increasingly higher levels of resistance.5,6 The wild-type and mutant enzymes can therefore be used as targets for screening of new anti-folates that would be effective against sensitive and resistant parasites, respectively. Such screening has been done through testing of individual candidate compounds against the enzymes.6–8

In the development of new anti-folates from combinatorial libraries, or from other types of libraries of potential inhibitors, it is desirable to screen them in groups to identify compounds with high affinities for the enzyme by screening through an iterative deconvolution technique9,10 and by pulsed ultrafiltration mass spectrometry.11–15 The basis of the method is to use the enzyme or protein receptor to retain the small molecules that bind tightly


References

in ultrafiltration and identify the retained compounds subsequently released using electro spray mass spectrometry. We now report a simplified ultrafiltration method for identification of tight-binding inhibitors of malarial dihydrofolate reductase, of the wild-type and mutant forms, based on binding of the enzyme present in amounts roughly equal to individual inhibitors. We show that the extent of binding of an inhibitor with the enzyme in competition with other compounds is related to its inhibition constant (Ki) and can be evaluated by measuring their binding in the filtrate fraction. Such Ki value for each component can be calculated and shown to be the same, with a small margin of error, as the Ks measured from the standard enzyme inhibition assay. In addition, we also incorporated a washing step in the protocol to disturb the binding equilibrium and thus allow for identification of those inhibitors with low dissociation (koff) rates in the retentate fraction. We could use this method for simple identification of lead candidates with high affinity (low Ki) and stability (low koff) from a mixture of compounds such as those obtained from combinatorial libraries for development of new anti-folate antimalarials.

**THEORY**

**General Protocol for Stoichiometric Selection.** The general protocol for stoichiometric selection of inhibitors by an enzyme is outlined in Figure 1. Tight-binding inhibitors can be selectively separated from other compounds in the library, based on their binding affinities to the enzyme. Following a brief incubation of the library with the reaction mixture containing the target enzyme, the unbound inhibitors can be separated from the reaction mixture by ultrafiltration into a filtrate for further identification and quantitation. A brief washing of the retentate fraction with the reaction buffer would disturb the binding equilibrium, and the complexes with lower stabilities (higher koff rates) would dissociate in greater amounts than those with higher stability in order to reach a new equilibrium. Some of the free inhibitors including those dissociated during the washing step are further removed with ultrafiltration. Subsequently, the remaining inhibitors in the retentate, both bound and unbound forms, can be analyzed after denaturation of the bound complexes with guanidine hydrochloride followed by another ultrafiltration. Inhibitors in each of these fractions can then be quantitated by HPLC based on their peak areas and identified by LC–MS. Inhibitors with higher stabilities (lower koff rates) are indicated by higher ratios of total inhibitor concentrations after and before the washing step.

**General Principle for Stoichiometric Selection.** In a mixture of an enzyme and several competitive inhibitors, the inhibitors would compete for the enzyme to form enzyme–inhibitor complexes. At equilibrium, the amount of each complex reflects the relative affinity of that inhibitor for the enzyme.

Assuming a simple 1:1 binding, an inhibitory constant Ki is defined as

\[ K_i = \frac{[E][I]}{[EI]} \]  

where [E], [I], and [EI] are concentrations of free enzyme, free inhibitor, and enzyme–inhibitor complex at equilibrium, respectively. In a mixture of several inhibitors, their bindings to the enzyme are competitive. So, the enzyme forms complexes in different amounts depending on affinity of individual inhibitor:

\[ E_i = \frac{[E] + [EI_1] + [EI_2] + [EI_3] + \cdots + [EI_n] + \cdots + [EI_n]}{[E] + [EI_1] + K_{i1} + [E][I_1] + K_{i2} + [E][I_2] + \cdots + [E][I_n] + K_{in}} \]

The total concentration of each inhibitor Ii is the sum of concentration of free inhibitor and its enzyme–inhibitor complex:

\[ I_i = [I_i] + [EI_i] \]

Therefore, knowing the total concentration of the enzyme and each inhibitor, the relative Ki of each inhibitor can be obtained through the measurement of free inhibitor concentrations and solving simultaneously eqs 6 for all inhibitors in the library.

**Calculation of Ki Values of Compounds in the Library.** A convenient and practical way for calculating the Ki value of each inhibitor is to solve simultaneously the following equations for all inhibitors in the library:

\[ I_i = [I_i] + \frac{E_i}{1 + \sum_{i=1}^{n} \frac{[I_i]}{K_{ii}}} \]
inhibitor is to find its value relative to that of an inhibitor with known $K_i$ value, such as Pyr ($K_{ipy}$), present in the same library as an internal standard (eq 7). The $K_i$ value can then be calculated from eq 8 without the need to know the concentration of free enzyme, $[E]$. This also helps to minimize any systematic errors that might arise in the method.

Relative $K_i$ value of inhibitor i,

$$
\frac{K_i}{K_{ipy}} = \frac{[E][I_i]}{[E][I_{ipy}]} \quad (7)
$$

$$
K_i = \frac{[I_i][E][I_{ipy}]}{[E][I_{ipy}]} K_{ipy} \quad (8)
$$

Alternatively and with higher accuracy, based on eq 3, if a set of compounds with known $K_i$ values is also included in the experiment, the concentration of free enzyme in each experiment can be calculated from a graph between $\log(K_i)$ and $\log([I_i]/[E])$, where the $y$ intercept equals $\log([E])$ (eq 9). Knowing the concentration of free enzyme, the $K_i$ value of the remaining inhibitors can be calculated individually through eq 3.

**MATERIALS AND METHODS**

**Construction of Combinatorial Libraries.** Pyr, Cyc, trimethoprim (Tmp), and a series of Pyr analogues were synthesized individually according to the methods described in our previous reports.\(^6\)\(^7\) Two libraries were created for stoichiometric selection. The first library, used for technique validation, was a mixture of Pyr, Cyc, and Tmp; and the other was a mixture of Pyr and 15 of its analogues. The construction of the latter library was based on $K_i$ values of each compound against wild-type and C59R strains.\(^6\)\(^7\) Selection of Tight-Binding Inhibitors. The combinatorial library was incubated with and without purified recombinant DHFRs in the presence of 100 $\mu$M NADPH for test and control, respectively. The reaction mixture of 500 $\mu$L contained 5 $\mu$M inhibitors, 50 $\mu$M PDHFRs in reaction buffer (20 mM potassium phosphate buffer, pH 7.0, 10 mM DTT, 0.1 mM EDTA, 50 mM KCl, 20% glycerol). After 5-min incubation at 25 °C, unbound compounds were separated by ultrafiltration through 10 kDa cutoff membrane (Centricon-10) using fixed angle centrifugation, at 4000g for 1 h at 25 °C. The filtrate of 350 $\mu$L was collected, and 100 $\mu$L was used for further analysis using HPLC. The peak area of each inhibitor was determined. Data were analyzed based on eq 9, where

$$
\% \text{ binding} = 100 \left(1 - \frac{\text{peak area of } [I_i] \text{ in the test}}{\text{peak area of } [I_i] \text{ in control}}\right)
$$

The retentate (150 $\mu$L) from the 500 $\mu$L reaction mixture ($v_0$) was washed with 1 mL of the reaction buffer by ultrafiltration. A portion of the washed retentate, 100 $\mu$L ($v_1$) from 250 $\mu$L ($v_0$), was treated with 500 $\mu$L of 6 M guanidine hydrochloride containing 12% DMSO ($v_2$) to release bound inhibitors from the enzyme. The released compounds were then separated from the enzyme by ultrafiltration. An aliquot of 100 $\mu$L ($v_3$) of released inhibitors was analyzed by HPLC. The peak area of each inhibitor was determined.

$$
\% \text{ binding} = 100 \left(\frac{v_4 \times \text{peak area of } [I_i] \text{ in the test}}{v_2 v_3 \times \text{peak area of } [I_i] \text{ in control}}\right)
$$

**Compounds Separation and Identification of Tight Binding Inhibitors.** Compounds in filtrate and retentate were separated by HPLC (Waters, Milford, MA, model Alliance 2690) with photodiode array using a reversed-phase column (Symmetry Shield, 5 $\mu$m, 4.6 × 250 mm, Waters) and a mixture of 8% acetonitrile, 10% methanol, and 25 mM ammonium acetate, pH 4.0, at a flow rate of 1 mL/min. The separation was started with the 8% acetonitrile mixture, followed by stepwise gradients of acetonitrile, 8–10, 10–14, 14–18, and 18–20% at 9–11, 30–32, 54–56, and 72–74 min of the run, respectively. The separation continued at 20% acetonitrile until the last compound was collected. The chromatogram was 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 masses, a mixture of the inhibitors with known concentrations was used to locate their peaks under the same separation protocol by HPLC.

**RESULTS AND DISCUSSION**

**Technique Validation.** To test whether PDHFR binds with a mixture of inhibitors according to theoretical expectation, the
Methods and analysed by HPLC as described in detail in Materials and Methods.

wild-type enzyme was titrated with an equimolar mixture each of Pyr, Cyc, and Tmp. Figure 2 shows that the concentrations of free inhibitors in the ultrafiltrate decreased sequentially in the order of Pyr, Cyc, and Tmp as expected. Assuming the value of 0.34 nM for $K_i$ of Pyr, the $K_i$ values for Cyc and Tmp were calculated to be 2.2 and 25.6 nM, respectively, using eq 8 and the data in Figure 2 at an enzyme/inhibitor ratio of 2 where all the inhibitors share the binding to the enzyme at less than 100% binding. These values are comparable with the previously reported $K_i$ values of 1.5 ± 0.3 and 10.3 ± 0.5 nM, respectively.5,7

This demonstrated that the principle of stoichiometric selection of inhibitors was applicable to PfDHFR and their inhibitors, and the $K_i$ values of tight-binding inhibitors in the library could be calculated reasonably accurately, so long as there were significant amounts of free and bound inhibitors for analysis.

Identification of Pyrimethamine Derivatives in the Library. A library of 16 Pyr derivatives was constructed based on their high binding affinities against wild-type PfDHFR as measured from conventional kinetics. The structures of these inhibitors in the library and their $K_i$s against plasmodial and human DHFRs are summarized in Table 1. These compounds in the library were separated by HPLC using a reversed-phase column (Symmetry Shield, RP18, 5 μm, 4.6 × 250 mm) and stepwise gradient mixture of 8%–20% acetonitrile, 10% methanol, and 25 mM ammonium acetate, pH 4.0. The amount of each inhibitor was determined by absorption at 254 nm. While P24 mixed with the buffer components at the solvent front, the other 15 compounds were completely separable from one another for further evaluation. Identification of compounds was based on the LC–MS results and retention time of each component. For those with the same masses, standard compounds were injected individually for determining their retention times.

Selection of Leads from the Library of Pyrimethamine Derivatives by the DHFRs. All inhibitors in the Pyr library were found to bind the enzyme to different extents in the binding assay using the excess molar ratio of 1.6:1.0 inhibitors to the DHFR (wild-type, double (C59R+S108N), and quadruple (S51I+C59R+S108N+I164L) PfDHFR mutant and human DHFR enzymes), i.e., 80 μM of total inhibitors (5 μM each) to 50 μM enzyme. Inhibitors with lower $K_i$ values (higher affinities) bound the enzyme to higher extents than those with higher $K_i$ values. Based on eq 9, a linear relationship is expected between log($K_i$) and log([I]/[EI]) from

### Table 1. Structure, Retention Time, Mass, and $K_i$ Value against DHFR of Each Pyrimethamine Analogue in the Pyrimethamine Library

<table>
<thead>
<tr>
<th>peak no.</th>
<th>substituents</th>
<th>retention time (min)</th>
<th>M + H$^+$ (m/z)</th>
<th>$K_i$, DHFR (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>calc</td>
<td>WT</td>
<td>double mutant</td>
</tr>
<tr>
<td>P20</td>
<td>H H H</td>
<td>CH$_3$CH$_3$</td>
<td>18.58 ± 0.98</td>
<td>2.3 ± 0.3$^a$</td>
</tr>
<tr>
<td>P15</td>
<td>H OCH$_2$O</td>
<td>CH$_3$CH$_3$</td>
<td>22.26 ± 1.35</td>
<td>1.1 ± 0.3$^a$</td>
</tr>
<tr>
<td>P26</td>
<td>H H H</td>
<td>(CH$_2$)$_2$COOCH$_3$</td>
<td>24.23 ± 1.62</td>
<td>0.6 ± 0.0$^a$</td>
</tr>
<tr>
<td>P18</td>
<td>H H OCH$_3$</td>
<td>CH$_3$CH$_3$</td>
<td>26.07 ± 1.74</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>P30</td>
<td>Cl H</td>
<td>CH$_3$CH$_3$</td>
<td>38.44 ± 1.37</td>
<td>0.8 ± 0.1$^a$</td>
</tr>
<tr>
<td>Pyr</td>
<td>H H Cl</td>
<td>CH$_3$CH$_3$</td>
<td>42.08 ± 1.72</td>
<td>0.3 ± 0.08</td>
</tr>
<tr>
<td>P29</td>
<td>Cl H</td>
<td>(CH$_2$)$_2$COOCH$_3$</td>
<td>44.84 ± 2.10</td>
<td>0.5 ± 0.09$^a$</td>
</tr>
<tr>
<td>P21</td>
<td>H Br</td>
<td>CH$_3$CH$_3$</td>
<td>49.06 ± 2.41</td>
<td>0.3 ± 0.0$^a$</td>
</tr>
<tr>
<td>P14</td>
<td>Cl Cl</td>
<td>CH$_3$CH$_3$</td>
<td>59.10 ± 2.18</td>
<td>12.6 ± 2.4$^a$</td>
</tr>
<tr>
<td>P12</td>
<td>H Cl</td>
<td>(CH$_2$)$_2$Cl</td>
<td>61.24 ± 1.85</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>P7</td>
<td>H Cl</td>
<td>CH$_3$CH$_3$</td>
<td>63.08 ± 1.93</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>P13</td>
<td>Cl Cl</td>
<td>CH$_3$CH$_3$</td>
<td>67.57 ± 2.38</td>
<td>1.0 ± 0.3$^a$</td>
</tr>
<tr>
<td>P4</td>
<td>H Cl</td>
<td>C$_6$H$_5$</td>
<td>74.14 ± 2.77</td>
<td>2.9 ± 0.6</td>
</tr>
<tr>
<td>P3</td>
<td>H Cl</td>
<td>CH$_2$CH(CH$_3$)$_2$</td>
<td>81.00 ± 2.60</td>
<td>0.3 ± 0.0</td>
</tr>
<tr>
<td>P5</td>
<td>H Cl</td>
<td>4OCH$_2$C$_6$H$_4$</td>
<td>85.14 ± 3.07</td>
<td>4.3 ± 0.7</td>
</tr>
</tbody>
</table>

$^a$ Data from ref 7. $^b$ Data from ref 8.
the filtrate fraction, which is confirmed experimentally with coefficient values ($r^2$) of 0.63 for wild-type PfDHFR, 0.92 for double- and quadruple-PfDHFR mutant enzymes, and 0.88 for human DHFR enzyme (data not shown).

**Analysis of Retentate Fraction.** While the results from filtrates prioritize inhibitors according to their relative binding affinities, results from the retentates further provide clues for whether the selected inhibitors possess high or low $k_{off}$ values to the enzyme. For those with low $K_i$ values, similar binding was obtained from filtrate and retentate fractions, whereas those with higher $K_i$ values tended to give higher ratios of [I]/[EI], presumably due to dissociation of weak-binding complexes in the ultrafiltration and the washing step. Brief washing of the retentates showed that while the inhibitors binding to the wild-type PfDHFR were still mostly retained, those binding to mutant PfDHFRs and the human enzyme dissociated significantly (Figure 3). The differences likely reflect poor stabilities (high $k_{off}$ rates) of these enzyme—inhibitors complexes. This phenomenon is obvious in the case of the quadruple mutant and human DHFRs, since these inhibitors were originally designed for wild-type PfDHFR based on molecular modeling.\(^7\,^8\,^15\) Compounds P13, P29, and P30 are among the inhibitors with low $K_i$ values against all the DHFRs tested. Although analysis of the filtrate fractions would classify these compounds as tight-binding inhibitors for both quadruple-mutant and human DHFRs, the retentate fractions showed that these compounds were easily released from the enzyme—inhibitor complexes following the brief washing step. The percentage of inhibitor binding was found to reduce by 40% compared with the filtrate fraction while only less than 20% were observed for the wild-type and double-mutant enzymes. These indicate that inhibitors in this library are not tight-binding inhibitors for quadruple PfDHFR and human DHFR when compared with the wild-type and double-mutant PfDHFR enzymes. Examination of retentate fraction is therefore useful for validating tight-binding inhibitors, which are good candidates for effective drugs.

**Calculation of $K_i$ Values of Compounds in the Library.** To evaluate whether this system can be used for calculating $K_i$ values of each inhibitor in the library simultaneously, the binding data of the inhibitors from the filtrate fraction were applied to eq 8 and compared to those measured kinetically.

When the data points of $K_i$ values higher than 2000 nM (relatively weak inhibitors) were omitted, the data gave a very good linear correlation was found between the $K_i$ values from both methods as shown in Figure 4. The $K_i$ values of all the compounds as calculated from the filtrate fractions based on eq 8 and those measured enzymatically. Data points were obtained from wild-type (●), double (■), and quadruple (▲) mutant PfDHFR, and (□) human DHFR.
good linear relationship with the $K_i$ measured with $r^2$ higher than 0.92. Statistical analysis using two-way ANOVA indicates no significant difference between the $K_i$ values calculated by the two methods. This simple method is therefore accurate in predicting $K_i$ values of compounds in combinatorial library.

**CONCLUSION**

We have shown that a library of inhibitors of PfDHFR, both wild-type and mutant enzymes responsible for drug resistance, can be selected by stoichiometric amounts of the enzyme. A simple ultrafiltration method was used, followed by analysis of both the filtrate and the washed retentate by HPLC. Both types of analysis gave comparable results for selection of tight-binding inhibitors while different results were obtained for those with moderate and weak affinities. Analysis of the filtrates alone provides data for calculating $K_i$ values precisely while combination of both analyses further differentiate inhibitors with strong and weak stabilities. This method should be applicable to selection of other anti-folate inhibitors of PfDHFR or, indeed, other enzymes that can be employed in stoichiometric amounts to select the tight-binding inhibitors.

The limitation to this technique is largely dependent on the solubility of the target enzyme, which is an important determining factor for the size of library and the concentrations of each inhibitor to be tested. In addition, the analysis of binding requires highly sensitive and sophisticated instrumentation like HPLC and intensive wash following each analysis. With appropriate analytical equipment, this method should be valuable in identifying the high-affinity leads from libraries of at least 15 and maybe up to 20 components depending on the solubility of the target enzyme and also in estimating the inhibition constants of the library members. Since our method provides a simple way to identify good lead candidates with high affinity (low $K_i$) and stability (low $k_{off}$) from a mixture of compounds, it is therefore useful as a primary screening of candidates for development of new effective drugs from compound libraries such as those obtained from combinatorial syntheses.

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