Structure–Activity Relationships and pH Dependence of Binding of 8-Alkyl-*N*5-deazapterins to Dihydrofolate Reductase

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Thermodynamic dissociation constants (K_d) have been determined for two series of 8-alkyl-*N*5-deazapterins in binary complexes with human and chicken dihydrofolate reductases (DHFRs) and ternary complexes with the enzyme·NADPH complex. For an initial series of 12 compounds with variable 8-alkyl substituents and pyrazine ring-methyl substitution patterns, K_d values at pH 6.6 were found to range from >100 to 0.5 μM, with consistent trends depending on the enzyme source, the size of the 8-substituent, and the presence and position of the pyrazine ring-methyl substituent. For most compounds in this first series, K_d values were significantly lower for the ternary complex than for the binary complex with ratios of K_d(binary)/K_d(ternary) ranging from 0.6 to 62, suggesting a degree of cooperativity in binding to the enzyme between ligand and cofactor. This effect was more pronounced for the human enzyme. The structure–activity relationships developed in the first series suggested a number of strategies for developing ligands with greater affinity for DHFR. These were tested with a second series of four compounds. The K_d of 80 nM at pH 6.6 of one of these compounds [5-methyl-8-isobutyl-*N*5-deazapterin (**15**)] in ternary complex with human DHFR is more than 200 times lower than that for the lead compound (8-methyl-*N*5-deazapterin (**1**); K_d 21 μM). Studies of binding stoichiometry indicated two binding sites in binary complexes with DHFR for 8-alkyl-N5-deazapterins with smaller 8-substituents. The second site was not found in ternary complexes or for ligands with larger 8-substituents, suggesting that the second ligand molecule in binary complexes is probably binding in the cofactor site and that the larger 8-substituents also bind in this area. A detailed study of the inhibition kinetics for one compound, 6,8-dimethyl-*N*5-deazapterin (**5**), showed it to be a competitive inhibitor of the chicken DHFR-catalyzed reduction of 6,8-dimethylpterin suggesting that the 8-alkyl-*N*5-deazapterins bind in the substrate site of DHFR. The pH dependence of the binding of several ligands in binary and ternary complexes with DHFR was examined by determining their K_d values at a range of pH's. This suggested that binding was predominantly between protonated ligand and deprotonated enzyme, but with variable contributions to binding observed between deprotonated enzyme and neutral ligand, and protonated enzyme and protonated ligand, depending on compound and complex type.

Introduction

The one-carbon metabolism of growing cells is critically dependent on the maintenance of adequate levels of tetrahydrofolate and its related cofactors. These cofactors act as one-carbon carriers in a range of reactions including *de novo* synthesis of both purines and pyrimidines.[1] In one of these reactions, deoxyuridylate is methylated to form thymidylate with the concomitant conversion of 5,10-methylenetetrahydrofolate to dihydrofolate. The restoration of the cellular pool of tetrahydrofolate and completion of the thymidylate cycle is achieved by the NADPH-mediated reduction of dihydrofolate which is catalyzed by dihydrofolate reductase (DHFR) (EC 1.5.1.3).[2] DHFR in mammalian cells also reduces folate to tetrahydrofolate, but at physiological pH this occurs at a greatly reduced rate compared with dihydrofolate reduction. Inhibition of DHFR results in cell stasis and death due to depletion of thymidylate,[3] and as this effect is more pronounced for rapidly proliferating cells with a high thymine requirement, these inhibitors have been used clinically to treat diseases such as malaria,[4] childhood leukemia,[5] and bacterial infections.[6]

The 8-alkyl-*N*5-deazapterins[7,8] are a new series of mechanism-based inhibitors of dihydrofolate reductase (DHFR) currently under development within our laboratory. Initial results for the biological activity with DHFR of some simply-substituted 8-alkyl-*N*5-deazapterins have been reported.[9,10] Here we report studies of the binding to DHFR of a number of 8-alkyl-*N*5-deazapterins with different patterns of 5-, 6-, and 7-methyl substitution and 8-substituents (Scheme 1). The structure–activity relationships developed from a first series of compounds have been used to design a second series with increased affinity for DHFR. The mode of binding of these ligands was examined by studying the pH dependence of binding and type of inhibition kinetics.

Chemistry

We have recently reported an improved procedure for the preparation of 8-alkyl-*N*5-deazapterins.[8] This involves the condensation in the presence of sodium bisulfite of a 2-amino-6-(alkylamino)pyrimidin-4(3*H*)-one with malonaldehyde bis(dimethyl acetal), methacrolein, methyl vinyl ketone, or crotonaldehyde to form 8-alkyl-*N*5-deazapterins, 6-methyl-8-alkyl-*N*5-deazapterins, 7-methyl-8-alkyl-*N*5-deazapterins, or 5-methyl-8-alkyl-*N*5-deazapterins, respectively. The ligand-

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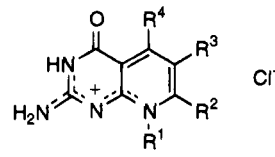
Scheme 1



	R^1	R^2	R^3	R^4
1	-CH_3	-H	-H	-H
2	-CH_2CH_3	-H	-H	-H
3	-$CH_2CH_2CH_3$	-H	-H	-H
4	-$CH(CH_3)_2$	-H	-H	-H
5	-CH_3	-H	-CH_3	-H
6	-CH_2CH_3	-H	-CH_3	-H
7	-$CH_2CH_2CH_3$	-H	-CH_3	-H
8	-$CH(CH_3)_2$	-H	-CH_3	-H
9	-CH_3	-CH_3	-H	-H
10	-$CH_2CH_2CH_3$	-CH_3	-H	-H
11	-CH_3	-H	-H	-CH_3
12	-$CH_2CH_2CH_3$	-H	-H	-CH_3
13	-$CH_2CH(CH_3)_2$	-H	-H	-H
14	-$CH_2C_6H_5$	-H	-H	-H
15	-$CH_2CH(CH_3)_2$	-H	-H	-CH_3
16	-$CH_2CH_2CH(CH_3)_2$	-H	-H	-CH_3
17	-H	-H	-CH_3	-H

design rationale, which has been outlined previously,[11] relies on the increased basicity of the 8-alkyl-*N*5-deazapterins (pK_a ~6.3–7.6[8]) compared with that for the *N*5-deazapterins (pK_a 4–5[8]) and the structurally-similar DHFR substrate, folate (pK_a 2.2[12]). The accepted mechanism for the reduction of folate requires an initial protonation on N8 of the substrate before hydride ion transfer.[13,14] On the basis of X-ray structures,[15] this protonated form is assumed[11] to bind to the enzyme *via* a salt bridge interaction between N3(*H*) and one of the 2-amino group protons and the carboxylate group of a conserved acidic residue in the enzyme active site (Glu 30 in mammalian DHFR). The increased basicity of the 8-alkyl-*N*5-deazapterins shifts the activity of these compounds into the physiological pH range[16] compared with folate for which the optimum activity occurs at pH ~4.[2]

Biological Studies

To examine the strength of binding of the 8-alkyl-*N*5-deazapterins we determined the thermodynamic dissociation constants with both human and chicken DHFRs. The dissociation constants for the equilibrium between free ligand and free enzyme with the enzyme–ligand complex were determined by a fluorescence titration procedure following the quenching of the native enzyme fluorescence due to ligand binding.

Birdsall *et al.*[17] studied the use of the fluorescence titration method for the determination of association constants between ligands and enzymes, in particular for DHFR, and related the ratio of bound to unbound enzyme in terms of the enzyme fluorescence according to eq 1:

$$\frac{[EL]}{[E_o]} = \frac{F_o - F}{F_o - F_s} \tag{1}$$

The equilibrium expression (eq 2) can then be solved for [EL] and an expression (eq 3) relating enzyme fluorescence F to $[E_o]$, $[L_o]$ and $K_{d(app)}$ derived.

$$E + L \rightleftharpoons EL \qquad K_{d(app)} = \frac{[E][L]}{[EL]} \tag{2}$$

where [E] = total free enzyme concentration, [L] = total free ligand concentration and [EL] = total enzyme-ligand complex concentration so that

$$F = [F_o - ((F_o - F_s)/2[E_o](C - (C^2 - 4[E_o][L_o])^{1/2}))] \tag{3}$$

where $C = [E_o] + [L_o] + K_d$ and $[L_o]$ = total ligand concentration, $[E_o]$ = total enzyme concentration, F_o = initial enzyme fluorescence, F_s = enzyme fluorescence at saturating ligand concentration, and K_d = apparent equilibrium dissociation constant.

This treatment assumes that all of the added ligand has equal affinity for the enzyme. However, for an ionizing ligand binding to an ionizable group of an enzyme, a number of different binding combinations are possible and each would be expected to have its own dissociation constant. Hence, $K_{d(app)}$ is merely an apparent binding constant which is a function of the dissociation constants for each of the possible enzyme–ligand complexes. This situation has been examined by Stone and Morrison[18] who derived a series of expressions for different binding models with $K_{d(app)}$ as a function of a single K_d for one of the enzyme–ligand complexes (defined as a pH-independent binding constant) and corresponding acid dissociation constants for the other enzyme–ligand complexes included in the model. In the following we present a revised treatment with expressions for $K_{d(app)}$ as a function of the individual dissociation constants for each combination of enzyme and ligand included in the model. These expressions can be used to evaluate directly the relative contributions to binding made by the different possible ligand–enzyme complexes whereas in the treatment of Stone and Morrison the K_d values for the different enzyme–ligand complexes are not obtained directly but must be derived from constants for each of the enzyme–ligand complexes which are termed "acid dissociation constants". These constants are not in our view true acid dissociation constants as their values do not reflect inherent acidities of these complexes, but rather they represent the relative stabilities of the complexes between different ionic forms of enzyme and ligand (Ivery, Jeong, and Gready, unpublished results).

The first model assumes that binding occurs only between protonated ligand and deprotonated enzyme:

$$E^- + LH^+ \rightleftharpoons E^-LH^+ \qquad K_d = \frac{[E^-][LH^+]}{[E^-LH^+]} \tag{4}$$

The K_d shown in eq 4 has been called a pH-independent dissociation constant. The apparent K_d can then be defined as follows:

$$K_{d(app)} = \frac{[E^- + EH][L + LH^+]}{[E^-LH^+]} \tag{5}$$

$$K_L = \frac{[L][H^+]}{[LH^+]} \tag{6}$$

$$K_E = \frac{[E^-][H^+]}{[EH]} \quad (7)$$

From eqs 4–7, an expression for $K_{d(app)}$ as a function of K_d, K_E, and K_L can be derived[18] as:

$$K_{d(app)} = K_d\left[1 + \frac{K_L}{[H^+]}\right]\left[1 + \frac{[H^+]}{K_E}\right] \quad (8)$$

Similarly, pH-independent K_d's for other enzyme–ligand complexes can be defined as:

$$E^- + L \rightleftharpoons E^-L \qquad K_{d(E-L)} = \frac{[E^-][L]}{[E^-L]} \quad (9)$$

$$EH + LH^+ \rightleftharpoons EHLH^+ \qquad K_{d(EHLH^+)} = \frac{[EH][LH^+]}{[EHLH^+]} \quad (10)$$

These equations can then be used to derive expressions for $K_{d(app)}$ for a number of binding models containing contributions from given enzyme–ligand complexes.

For E^-LH^+ and E^-L

$$K_{d(app)} = \frac{[E^- + EH][L + LH^+]}{[E^-LH^+ + E^-L]} \quad (11)$$

which may be re-expressed as:

$$K_{d(app)} = \frac{\left[1 + \frac{K_L}{[H^+]}\right]\left[1 + \frac{[H^+]}{K_E}\right]}{\left[\frac{1}{K_d} + \frac{K_L}{K_{d(E-L)}[H^+]}\right]} \quad (12)$$

For $EHLH^+$ and E^-LH^+

$$K_{d(app)} = \frac{[E^- + EH][L + LH^+]}{[E^-LH^+ + EHLH^+]} \quad (13)$$

which may be re-expressed as:

$$K_{d(app)} = \frac{\left[1 + \frac{K_L}{[H^+]}\right]\left[1 + \frac{[H^+]}{K_E}\right]}{\left[\frac{1}{K_d} + \frac{[H^+]}{K_{d(EHLH^+)}K_E}\right]} \quad (14)$$

For $EHLH^+$, E^-LH^+, and E^-L

$$K_{d(app)} = \frac{[E^- + EH][L + LH^+]}{[E^-LH^+ + E^-L + EHLH^+]} \quad (15)$$

which may be re-expressed as:

$$K_{d(app)} = \frac{\left[1 + \frac{K_L}{[H^+]}\right]\left[1 + \frac{[H^+]}{K_E}\right]}{\left[\frac{1}{K_d} + \frac{[H^+]}{K_{d(EHLH^+)}K_E} + \frac{K_L}{K_{d(E-L)}[H^+]}\right]} \quad (16)$$

By determining the apparent dissociation constants for the binding of a number of 8-alkyl-*N*5-deazapterins to DHFR at various pH's and examining the fit of the data to these equations, we will be able to evaluate the relative contributions of the different ionic forms of the ligand and enzyme and how different compound substitution patterns affect these contributions.

Results and Discussion

Structure–Activity Relationships. Previous studies[10,19] on the biological activity of the inhibitor 6,8-dimethyl-*N*5-deazapterin (**5**) and the related substrates, the 8-alkylpterins,[11,20] have shown that the binding of these ligands to DHFR is strongly pH-dependent with optimal binding generally found in the region of the pK_a of the ligand. As the number of ligands in our first series of compounds (Scheme 1, compounds **1**–**12**) precluded full pH profiles for each, the binding assays were performed at pH 6.6. This pH is close to the pK_a of most of the ligands, except the 5-methyl-substituted compounds ($pK_a \sim 7.3$–7.6), and hence was expected to allow a good estimate for the strongest binding of each ligand. Ligand dissociation constants were measured for both binary and ternary complexes with both chicken liver and recombinant human DHFRs and used as the measure of binding for developing structure–activity relationships. K_d's are easier to measure than inhibition constants and also are a direct thermodynamic measure of binding which may be compared with theoretical calculations.[21]

Table 1 lists the $K_{d(app)}$ values for all the ligands in the first series (Scheme 1, **1**–**12**). All ligands showed measurable binding to DHFR with $K_{d(app)}$ values ranging from 138 ± 28 μM for 6-methyl-8-propyl-*N*5-deazapterin (**7**) in binary complex with chicken enzyme to 0.5 ± 0.1 μM for 6,8-dimethyl-*N*5-deazapterin (**5**) in ternary complex with human enzyme. Given the range of sizes of the 8-substituents investigated in this series (methyl to isopropyl), these data suggest that DHFR has considerable flexibility in accommodating the 8-substituent. As discussed below, there are a number of specific trends in the binding data for the different groups of ligands.

8-Alkyl-*N*5-deazapterins with No Ring-Methyl Substituent (1–4). Generally these ligands show increased affinity for DHFR as the size of the 8-substituent increases. The trend for binary complexes of the chicken enzyme shows $K_{d(app)}$ values decreasing from 58 ± 5 μM for **1** (8-methyl) to 16 ± 1 for **4** (8-isopropyl). Similarly, the $K_{d(app)}$ values for the binary complexes with the human enzyme decrease from 118 ± 12 μM for **1** (8-methyl) to 12.0 ± 0.1 μM for **3** (8-propyl). However, the binding of **4** (8-isopropyl) in the binary complex is significantly weaker (34 ± 2 μM) than the straightforward trend would suggest.

For the ternary complex of 8-alkyl-*N*5-deazapterins with NADPH and DHFR the strength of binding again increases as the size of the 8-substituent increases, and in all cases the K_d values for the ternary complexes are lower than those for the same ligands in the binary complex. Tighter binding of ligands in ternary complex with DHFR compared with the binary complex has been observed previously in binding of NADPH and its analogues with DHFR complexed with different inhibitors,[22,23] methotrexate in the ternary complex with *E.*

Table 1. Apparent Dissociation Constants $K_{d(app)}$ (μM)[a] at pH 6.6 for an Initial Series of 8-Alkyl-*N*5-deazapterins in Binary and Ternary Complexes with Cofactor NADPH and Chicken and Human DHFRs

	chicken DHFR			human DHFR		
compound	no NADPH	with NADPH	ratio[b]	no NADPH	with NADPH	ratio[b]
1 (8-methyl)	58 ± 5	21 ± 1	2.8	118 ± 12	21 ± 2	5.6
2 (8-ethyl)	38 ± 3	32 ± 6	1.2	60 ± 2	13 ± 2	4.6
3 (8-propyl)	18 ± 1	11 ± 1	1.6	12.0 ± 0.1	4.0 ± 0.7	3.0
4 (8-isopropyl)	16 ± 1	10 ± 1	1.6	34 ± 2	1.9 ± 0.2	17.9
5 (6,8-dimethyl)	16 ± 1	1.1 ± 0.1	14.5	31 ± 1	0.5 ± 0.1	62
6 (6-methyl-8-ethyl)	60 ± 6	74 ± 8	0.8	130 ± 9	16 ± 2	8.1
7 (6-methyl-8-propyl)	138 ± 28	25 ± 3	5.5	108 ± 7	4.6 ± 0.5	23.5
8 (6-methyl-8-isopropyl)	44 ± 2	23 ± 3	1.9	60 ± 4	18 ± 1	3.3
9 (7,8-dimethyl)	11 ± 1	1.1 ± 0.1	10	21 ± 1	2.9 ± 0.3	7.2
10 (7-methyl-8-propyl)	3.4 ± 0.1	5.9 ± 0.5	0.6	3.4 ± 0.2	2.0 ± 0.3	1.7
11 (5,8-dimethyl)	20 ± 1	11 ± 2	1.8	21 ± 1	5.1 ± 0.7	4.1
12 (5-methyl-8-propyl)	7.5 ± 0.7	5.5 ± 0.4	1.4	7.1 ± 0.2	1.2 ± 0.2	5.9

[a] Standard errors. [b] Ratio of K_d's for binary to ternary complexes.

coli DHFR and NADPH,[24] and also for folate fragments binding in combination as compared with their binding alone.[25]

The ability of ligands to assist each other in binding to an enzyme is termed cooperativity, and the degree of cooperativity exhibited by a pair of ligands is defined as the ratio of the binary complex K_d to the ternary complex K_d.[2] For the 8-alkyl-*N*5-deazapterins these values are shown in Table 1 and indicate that cooperativity induced by NADPH in binding to chicken DHFR ranges from 2.8 to 1.2 while for human DHFR it ranges from 17.9 to 3.0. For the chicken enzyme the extent of cooperativity induced by NADPH for a particular ligand may be seen to decrease as the size of the 8-substituent is increased, while human enzyme appears to be able to accommodate the larger substituents and NADPH more effectively. This results in the human enzyme having tighter binding than the chicken enzyme for all the ligands in the ternary complex with NADPH.

8-Alkyl-*N*5-deazapterins with a 6-Methyl Substituent (5–8). The $K_{d(app)}$ data for binary complexes of the 6-methyl compounds show significant differences from that for the 8-alkyl-*N*5-deazapterins with binding to both enzymes generally becoming weaker as the size of the 8-substituent increases. For the ternary complexes, significant cooperativity is observed again for most compounds with both enzymes. This cooperativity is particularly pronounced for **5** (6,8-dimethyl; 15 or 62) which has the lowest K_d value for the first series of compounds with both the chicken and human enzymes: 1.1 and 0.5 μM, respectively. However, for the chicken enzyme the degree of cooperativity for the larger 8-substituents is again considerably less than that observed for the human enzyme with binding for compounds **2–4** being considerably weaker than for human enzyme. In general these results suggest that both enzymes have difficulty in accommodating both a 6-methyl and a large 8-substituent but that, as for the 8-substituted compounds, the human enzyme has greater flexibility in accommodating both the larger ligands and cofactor in the active site.

8-Alkyl-*N*5-deazapterins with 7-Methyl Substituent (9–10). While the $K_{d(app)}$ values for 7,8-dimethyl-*N*5-deazapterin (**9**) with chicken DHFR are similar to those for 6,8-dimethyl-*N*5-deazapterin (**5**), for binding with human DHFR the cooperativity for **9** is markedly lower than for **5** resulting in a significantly higher K_d value in the ternary complex. For 7-methyl-8-propyl-*N*5-deazapterin (**10**) strong binding is observed to both enzymes in the binary complex, but binding in the ternary complex is actually weakened with chicken enzyme and only weakly increased with human enzyme. This suggests that both enzymes have significant difficulty in accommodating a large 8-substituent, a 7-methyl group, and NADPH cofactor, but that the human enzyme again has more active site flexibility.

8-Alkyl-*N*5-deazapterins with a 5-Methyl Substituent (11–12). Both the 8-methyl- (**11**) and 8-propyl- (**12**) 5-methyl-substituted compounds bind well to both enzymes with significant cooperativity in binding in the ternary complexes. Again the cooperativity for the 8-propyl compound was significantly greater with the human enzyme (5.9) than with the chicken enzyme (1.4). Comparing the ternary complex $K_{d(app)}$ values for compounds **1** and **11** and **3** and **12** indicates that introducing a 5-methyl substituent strengthens binding by a factor of ~2 for the chicken enzyme and ~4 for the human enzyme for both large and small 8-substituents.

Summary of Structure–Activity Relationships. These patterns may be summarized as follows:

The binding activity of 8-alkyl-*N*5-deazapterins depends strongly on both substituent pattern, enzyme source and the presence or absence of cofactor.

Binding is generally significantly stronger for ternary complexes.

Increasing the size of the 8-substituent generally increases the strength of binding.

Addition of a 6-methyl substituent greatly strengthens the binding for compounds with a small 8-substituent but either weakens or leaves unchanged the binding of compounds with larger 8-substituents.

Addition of a 5-methyl substituent strengthens the binding with both enzymes for compounds with both small and large 8-substituents.

Strategies for Refinement of Compound Activity. From this analysis of the structure–activity relationships for the first series of compounds, a number of strategies for further developing these compounds were devised:

Optimization of the size and shape of the 8-substituent.

Optimization of the structure of the 6-substituent coupled with an 8-methyl- substituent.

Optimization of the 8-substituent coupled with an optimum 5-substituent.

Second Ligand Series. As a start to examining these design strategies, compounds **13–16** were prepared. Compounds **13** (8-isobutyl) and **14** (8-benzyl)

Table 2. Apparent Dissociation Constants $K_{d(app)}$ (μM)[a] at pH 6.6 for the Second Series of 8-Alkyl-*N*5-deazapterins for Binary and Ternary Complexes with Cofactor NADPH and Chicken and Human DHFRs

	chicken DHFR			human DHFR		
compound	no NADPH	with NADPH	ratio[b]	no NADPH	with NADPH	ratio[b]
13 (8-isobutyl)	2.5 ± 0.1	2.2 ± 0.2	1.1	2.2 ± 0.2	0.60 ± 0.10	3.7
14 (8-benzyl)	3.7 ± 0.2	7.3 ± 0.9	0.5	1.7 ± 0.1	0.70 ± 0.06	2.4
15 (5-methyl-8-isobutyl)	3.5 ± 0.2	1.6 ± 0.1	2.2	2.4 ± 0.1	0.08 ± 0.02	30
16 (5-methyl-8-isoamyl)	3.3 ± 0.2	4.5 ± 0.3	0.7	1.8 ± 0.1	0.70 ± 0.07	2.6

[a] Standard errors. [b] Ratio of K_d's for binary to ternary complexes.

further test the capacity of the 8-substituent binding site while compound **15** (5-methyl-8-isobutyl) tests whether, and to what extent, a 5-methyl group would strengthen binding of a larger 8-substituent. Compound **16** (5-methyl-8-isoamyl) represents dual changes of extending the size of the 8-substituent and adding a 5-methyl group.

Binding data for these compounds are listed in Table 2. It is apparent that all compounds show quite strong and similar binding to both enzymes in the binary complex with the lowest K_d values being found for **14** and **16** with human enzyme. However, the ternary complexes show considerable differences in binding and particular differences between the enzymes. For the chicken enzyme only **15** shows significant cooperativity (~2) in binding: both **14** and **16** showing significantly weakened binding in the presence of NADPH, and the binding of **13** is essentially unchanged. These results suggest that the active-site capacity of the chicken enzyme has been exceeded by the large benzyl and isoamyl groups of **14** and **16**, respectively. For the human enzyme, the cooperativity in binding is again significantly greater than for the chicken enzyme. Compounds **13**, **14**, and **16** all show cooperativity of ~3 with K_d values of ~0.7 μM. However, 5-methyl-8-isobutyl-*N*5-deazapterin shows very strong cooperativity in binding of 30 times to give a K_d value of 0.08 μM. This value was determined using a fluorimetric titration procedure which followed the quenching of the ligand fluorescence on binding. It was necessary to modify the standard procedure following the quenching of the enzyme fluorescence when attempts to measure the K_d proved unreliable due to poor sensitivity resulting from the low enzyme concentration necessary to assay low K_d values. This value is more than 2 orders of magnitude lower than the lead compound **1** (8-methyl) and, given the relatively simple modifications reported, suggests that the 8-alkyl-*N*5-deazapterins have good potential for the development of very strong inhibitors of DHFR.

Interestingly, the strong binding of 6,8-dimethyl-*N*5-deazapterin (**5**) and 5-methyl-8-isobutyl-*N*5-deazapterin (**15**) in the ternary complex with human enzyme are both characterized by very strong cooperativity in binding of 62 and 30 times, respectively. Elucidation of the structural factors enhancing the cooperativity may provide useful hints for future design.

pH Dependence of Binding of 8-Alkyl-*N*5-deazapterins. As outlined, the design rationale for the 8-alkyl-*N*5-deazapterins assumes that the ligand binds to the enzyme in the protonated form. From this hypothesis it is expected that the enzyme binding of these ligands would be strongly pH-dependent, and this has been observed[19] for the binding of 6,8-dimethyl-*N*5-deazapterin (**5**) in ternary complex with human enzyme and NADPH. The $K_{d(app)}$ data for **5** for a range of pH's were fitted to eq 8, and a value for the dissociation constant of the complex between protonated ligand and deprotonated enzyme (K_d, eq 4) was estimated.[19] These results suggested that the ligands are binding in the designed mode.

To examine further the nature of the binding of the 8-alkyl-*N*5-deazapterins we have studied the pH dependence of binding of 8-propyl- (**3**), 7-methyl-8-propyl- (**10**) and 5-methyl-8-isobutyl-*N*5-deazapterins (**15**) in both binary and ternary (NADPH) complexes with human DHFR and 5-methyl-8-propyl-*N*5-deazapterin (**12**) in binary and ternary complexes with chicken DHFR. Figures 1 and 2 show the data for each compound in the binary and ternary complexes, respectively, with data fitting to eq 8 representing the enzyme–ligand complex model E^-LH^+. Inspection of these figures shows apparently good fits to the model in some cases, but for others the data clearly do not fit the model. Table 3 summarizes the fit parameters obtained for each compound. These parameters are the acid dissociation constants for the ligand and enzyme and a value for the dissociation constant of the protonated ligand–deprotonated enzyme complex (K_d, eq 4). In all cases the fitted pK_L value is significantly higher than that measured directly for each ligand.[8] While previous authors have attributed these differences to experimental error,[26] the discrepancies in Table 3 appear to us to be too large to be explained in this manner. The predicted values for pK_E also are considerably lower than would be expected as this ionization has been estimated previously to have a pK of ~6.[26-30] Also, the K_d values predicted from these fits are extremely low compared with the apparent K_d values. All these findings suggest that our data is not well represented by the simple model of eq 8.

These higher than expected estimated values for pK_L given by the simple E^-LH^+ model suggest that some binding may be occurring between E^- and L. To examine this possibility we have fitted the data from Figures 1 and 2 to eq 12 which represents binding for both an E^-LH^+ and E^-L complex (eq 11); the fitted parameters are given in Table 4. These parameters indicate a better estimation of pK_L is obtained with this model for all cases compared with the directly-measured experimental values (Table 3). For the 8-propyl (**3**) and 5-methyl-8-isobutyl (**15**) compounds the estimated value for pK_L in both complexes now agrees with the experimental value within the fit standard errors. The estimated value for the 7-methyl-8-propyl (**10**) compound in the ternary complex and 5-methyl-8-propyl-*N*5-deazapterin (**12**) for the binary complex also fit within the estimated errors, but for **10** and **12** in the binary and ternary complexes, respectively, the estimates differs by ~0.4 and ~0.7 from the experimental values. The estimated values for pK_E are now closer to their expected values, especially for the ternary com-

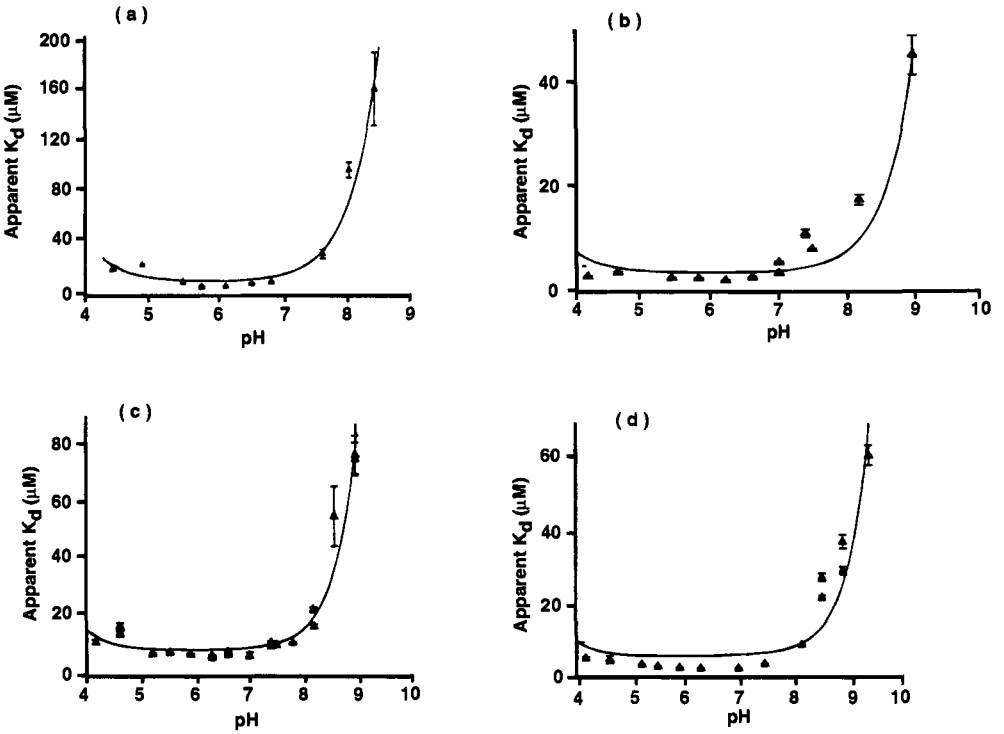


Figure 1. Plots of apparent thermodynamic dissociation constant ($K_{d(app)}$) versus pH for binary complexes of: (a) 8-propyl-*N*5-deazapterin (**3**) with human DHFR; (b) 7-methyl-8-propyl-*N*5-deazapterin (**10**) with human DHFR; (c) 5-methyl-8-propyl-*N*5-deazapterin (**12**) with chicken DHFR; and (d) 5-methyl-8-isobutyl-*N*5-deazapterin (**15**) with human DHFR. The curve in each plot represents the best fit for data to a binding model with only one enzyme–ligand complex (E^-LH^+) represented by eq 8.

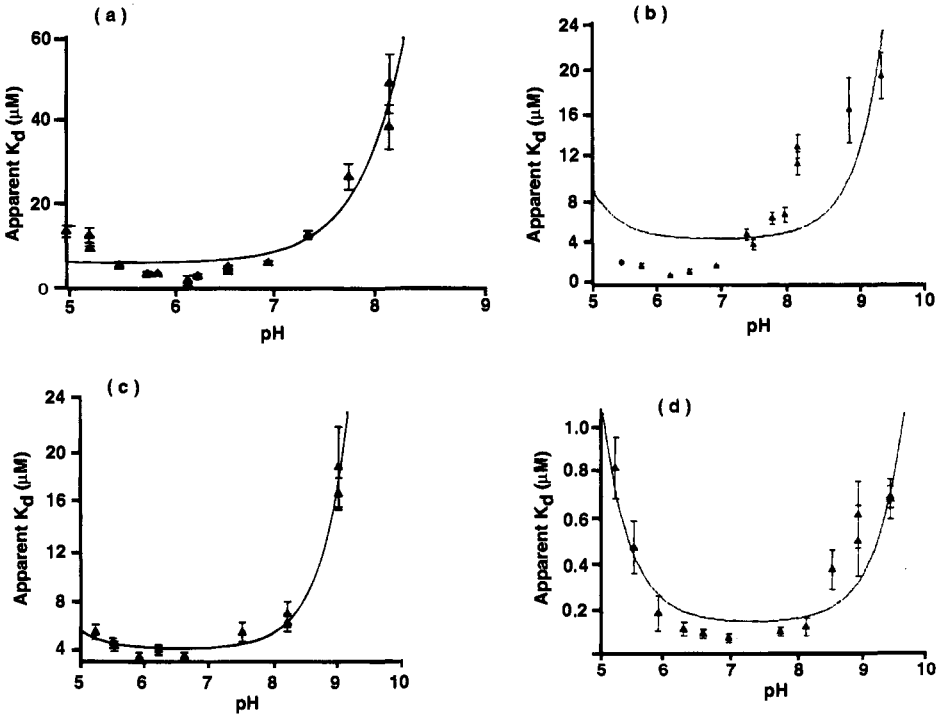


Figure 2. Plots of apparent thermodynamic dissociation constant ($K_{d(app)}$) versus pH for ternary complexes of: (a) 8-propyl-*N*5-deazapterin (**3**) with human DHFR; (b) 7-methyl-8-propyl-*N*5-deazapterin (**10**) with human DHFR; (c) 5-methyl-8-propyl-*N*5-deazapterin (**12**) with chicken DHFR; and (d) 5-methyl-8-isobutyl-*N*5-deazapterin (**15**) with human DHFR. The curve in each plot represents the best fit for data to a binding model with only one enzyme–ligand complex (E^-LH^+) represented by eq 8.

plexes of **3**, **10**, and **15** where estimates of pK_E are above 6. Table 4 also shows much more realistic values of K_d with these values now of similar magnitude to the lowest $K_{d(app)}$ value determined. These results suggest that this model is a good representation for binding in the ternary complex.

For the binary complexes it may be seen that while estimates of both pK_L and K_d have improved, the estimates of pK_E are still too low compared with previous estimates of this parameter. Examination of the $K_{d(app)}$ data for each compound in Figure 1 suggests that as the pH was lowered binding initially began to weaken but then plateaued. This suggests a possible contribution to binding by interactions between proton-

Table 3. Fitting of Data for the pH Dependence of the Apparent Dissociation Constants $K_{d(app)}$ by Eq 8 to a Binding Model (Eq 4) with Only E^-LH^+ as the Enzyme–Ligand Complex Involved in Ligand Binding

compound	complex	enzyme	K_d (μM)[a]	pK_L (expt)[b]	pK_L[a]	pK_E[a]
3 (8-propyl)	binary	human	0.0207 ± 0.003	6.51 ± 0.03	7.06 ± 0.08	3.90 ± 0.11
3 (8-propyl)	ternary	human	0.0026 ± 0.0011	6.51 ± 0.03	7.40 ± 0.12	3.64 ± 0.20
10 (7-methyl-8-propyl)	binary	human	0.0005 ± 0.0003	6.86 ± 0.03	7.95 ± 0.14	4.04 ± 0.22
10 (7-methyl-8-propyl)	ternary	human	0.0006 ± 0.0003	6.86 ± 0.03	8.39 ± 0.17	4.62 ± 0.34
12 (5-methyl-8-propyl)	binary	chicken	0.0006 ± 0.0004	7.52 ± 0.02	8.09 ± 0.08	3.92 ± 0.33
12 (5-methyl-8-propyl)	ternary	chicken	0.0005 ± 0.0003	7.52 ± 0.02	8.47 ± 0.05	4.59 ± 0.23
15 (5-methyl-8-isobutyl)	binary	human	0.0002 ± 0.0001	7.62 ± 0.04	8.47 ± 0.17	3.90 ± 0.25
15 (5-methyl-8-isobutyl)	ternary	human	0.0001 ± 0.0001	7.62 ± 0.04	8.89 ± 0.26	5.84 ± 0.27

[a] See eq 8 for definitions of $K_d = K_{d(ELH)}$, pK_L and pK_E. [b] Experimental pK_a's from ref 8.

Table 4. Fitting of Data for the pH Dependence of the Apparent Dissociation Constants $K_{d(app)}$ by Eq 12 to a Binding Model (Eq 11) Including Formation of Both E^-LH^+ and E^-L Complexes Involved in Ligand Binding

compound	complex	enzyme	K_d (μM)[a]	$K_{d(EL)}$ (μM)[a]	pK_L[a]	pK_E[a]
3 (8-propyl)	binary	human	6.5 ± 3.9	407 ± 123	6.56 ± 0.31	4.62 ± 0.41
3 (8-propyl)	ternary	human	0.83 ± 1.01	85 ± 16	6.20 ± 0.56	6.20 ± 0.56
10 (7-methyl-8-propyl)	binary	human	2.7 ± 0.4	69 ± 4	7.28 ± 0.09	3.58 ± 0.61
10 (7-methyl-8-propyl)	ternary	human	0.45 ± 1.33	20 ± 1	6.48 ± 1.32	6.00 ± 1.59
12 (5-methyl-8-propyl)	binary	chicken	6.9 ± 1.4	164 ± 36	7.69 ± 0.14	4.29 ± 0.24
12 (5-methyl-8-propyl)	ternary	chicken	3.7 ± 0.5	52 ± 35	8.24 ± 0.20	4.86 ± 0.30
15 (5-methyl-8-isobutyl)	binary	human	3.0 ± 1.0	89 ± 10	7.73 ± 0.17	4.12 ± 0.44
15 (5-methyl-8-isobutyl)	ternary	human	0.030 ± 0.030	0.86 ± 0.06	7.31 ± 0.53	6.67 ± 0.49

[a] See eq 12 for definitions of $K_d = K_{d(ELH)}$, $K_{d(EL)}$, pK_L and pK_E.

Table 5. Fitting of Data for the pH Dependence of the Apparent Dissociation Constants $K_{d(app)}$ by Eq 16 to a Binding Model (Eq 15) Including Formation of $EHLH^+$, E^-LH^+, and E^-L Complexes Involved in Ligand Binding

compound	complex	enzyme	K_d (μM)[a]	$K_{d(EHLH)}$ (μM)[a]	$K_{d(EL)}$ (μM)[a]	pK_L[a]	pK_E[a]
3 (8-propyl)	binary	human	2.1 ± 7.8	29 ± 11	387 ± 86	6.01 ± 1.75	6.03 ± 1.72
10 (7-methyl-8-propyl)	binary	human	2.8 ± 0.6	3.6 ± 0.9	68 ± 4	7.29 ± 0.14	3.72 ± 2.48
12 (5-methyl-8-propyl)	binary	chicken	5.3 ± 1.9	16 ± 4	153 ± 26	7.53 ± 0.22	5.61 ± 0.72
15 (5-methyl-8-isobutyl)	binary	human	2.2 ± 1.7	5.9 ± 2.4	87 ± 10	7.57 ± 0.41	5.47 ± 1.34

[a] See eq 16 for definitions of $K_d = K_{d(ELH)}$, $K_{d(EHLH)}$, $K_{d(EL)}$, pK_L, and pK_E.

Table 6. Fitting of Data for the pH Dependence of the Apparent Dissociation Constants $K_{d(app)}$[a]

compound	complex	enzyme	K_d (μM)[b]	$K_{d(EHLH)}$ (μM)[b]	$K_{d(EL)}$ (μM)[b]	pK_L[b]	pK_E[b]
3 (8-propyl)	binary	human	5.8 ± 0.7	29 ± 16	420 ± 96	6.51 (fixed)	5.48 ± 0.56
3 (8-propyl)	ternary	human	1.7 ± 0.2		89 ± 17	6.51 (fixed)	5.88 ± 0.10
10 (7-methyl-8-propyl)	binary	human	1.2 ± 0.1	3.7 ± 0.9	64 ± 3	6.86 (fixed)	5.90 (fixed)[c]
10 (7-methyl-8-propyl)	ternary	human	1.0 ± 0.1		20 ± 1	6.86 (fixed)	5.48 ± 0.42
12 (5-methyl-8-propyl)	binary	chicken	5.1 ± 0.5	16 ± 4	150 ± 19	7.52 (fixed)	5.65 ± 0.48
12 (5-methyl-8-propyl)	ternary	chicken	2.4 ± 0.4		22.5 ± 1.9	7.52 (fixed)	5.42 ± 0.19
15 (5-methyl-8-isobutyl)	binary	human	2.6 ± 0.2	6.0 ± 3.2	89 ± 8	7.66 (fixed)	5.25 ± 0.95
15 (5-methyl-8-isobutyl)	ternary	human	0.057 ± 0.008		0.88 ± 0.06	7.66 (fixed)	6.37 ± 0.07

[a] For binary complexes to a binding model including E^-LH^+ and an E^-L complexes (eqs 11 and 12) and for ternary complexes to a model including $EHLH^+$, E^-LH^+, E^-L complexes (eqs 15 and 16) with pK_L value for the ligand fixed at the independently-determined experimental value. [b] See eqs 12 and 16 for definitions of $K_d = K_{d(ELH)}$, $K_{d(EHLH)}$, $K_{d(EL)}$, pK_L, and pK_E. [c] Fixed value: see text.

ated enzyme and protonated ligand. Table 5 shows fit parameters obtained when $K_{d(app)}$ data for the binary complexes were fitted by eq 16 to a model involving three enzyme–ligand complexes ($EHLH^+$, E^-LH^+, E^-L; eq 15). The estimates for pK_E for the 8-propyl, 5-methyl-8-propyl and 5-methyl-8-isobutyl compounds have now increased to between ~5.5 and 6. For 7-methyl-8-propyl-*N*5-deazapterin, however, the estimated pK_E value is still very low. This may be due to the relatively low dissociation constant for the $EHLH^+$ complex (3.6 μM) which makes it difficult to ascertain the pK_E of the enzyme.

In summary, the ternary complex data are best represented by a model including an E^-LH^+ and an E^-L complex while the data for the binary complexes are best represented by a model including $EHLH^+$, E^-LH^+ and E^-L complexes. However, examination of the data in Tables 4 and 5 indicates very large errors in the estimates of many of the parameters even though comparison of the experimental data with the fitted curves (not shown) shows quite good agreement. This probably results from compounding of errors in fitting multiple parameters in these equations. To check this possibility we repeated the fits for the best models for each compound but fixed the pK_L for each ligand at the independently-determined value (Table 3).

For 7-methyl-8-propyl-*N*5-deazapterin the pK_E value was also fixed at 5.9. The fitted parameters are shown in Table 6, and fitted plots of the experimental data to the relevant models are shown in Figures 3 and 4 for the binary and ternary complexes, respectively. The curves in Figures 3 and 4 show very good agreement with the experimental data, and the fit parameters in Table 6 now show errors generally below ~20%. The estimates of pK_E for the human enzyme shown in Table 4 range from 5.25 ± 0.95 to 6.37 ± 0.07 with an average value of ~5.70. This value compares favorably with a previous estimate[19] of 5.9.

Comparison of the dissociation constants for the E^-L complex ($K_{d(EL)}$) indicates that for all compounds the neutral ligand binds more strongly to the enzyme in the ternary complex than in the binary complex. Further-

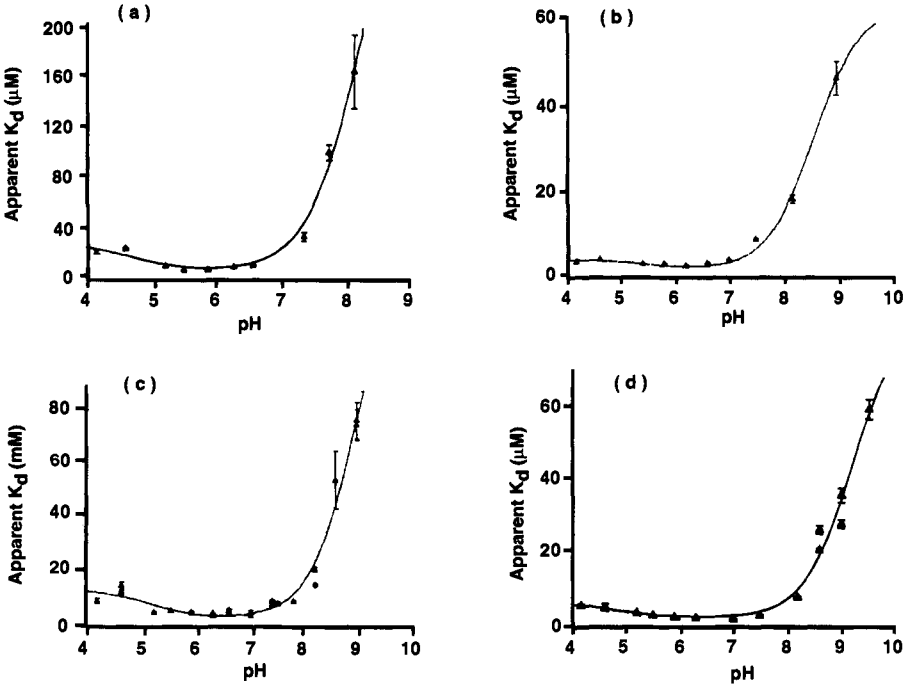


Figure 3. Plots of apparent thermodynamic dissociation constant ($K_{d(app)}$) versus pH for binary complexes of: (a) 8-propyl-*N*5-deazapterin (**3**) with human DHFR; (b) 7-methyl-8-propyl-*N*5-deazapterin (**10**) with human DHFR; (c) 5-methyl-8-propyl-*N*5-deazapterin (**12**) with chicken DHFR; and (d) 5-methyl-8-isobutyl-*N*5-deazapterin (**15**) with human DHFR. The curve in each plot represents the best fit to a binding model with three enzyme–ligand complexes ($EHLH^+$, E^-LH^+, E^-L) represented by eq 16. For each fit the ligand pK_a was fixed to the independently-determined value and for 7-methyl-8-propyl-*N*5-deazapterin pK_E was fixed to 5.9.

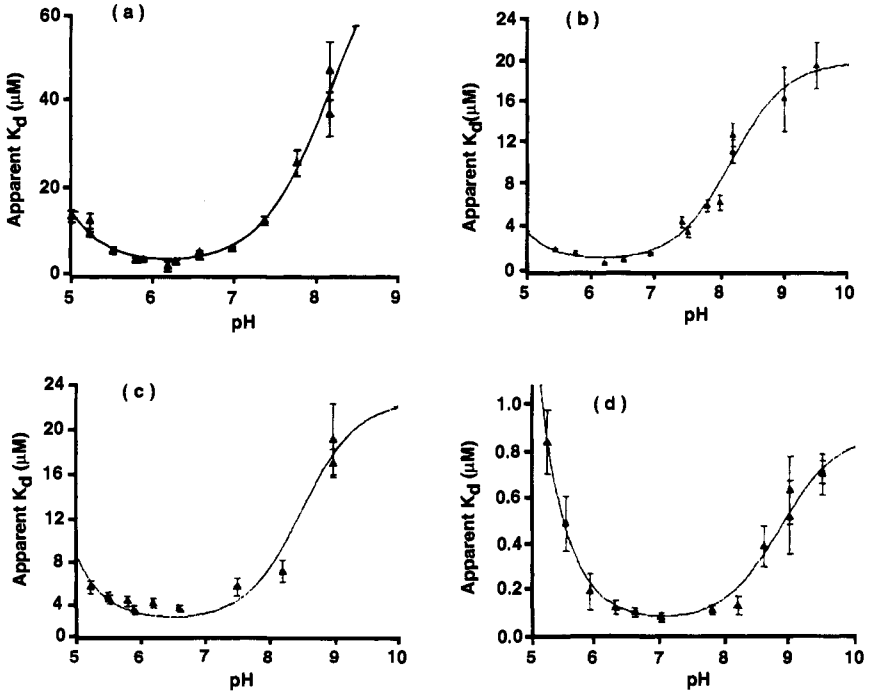


Figure 4. Plots of apparent thermodynamic dissociation constant ($K_{d(app)}$) versus pH for ternary complexes of: (a) 8-propyl-*N*5-deazapterin (**3**) with human DHFR; (b) 7-methyl-8-propyl-*N*5-deazapterin (**10**) with human DHFR; (c) 5-methyl-8-propyl-*N*5-deazapterin (**12**) with chicken DHFR; and (d) 5-methyl-8-isobutyl-*N*5-deazapterin (**15**) with human DHFR. The curve in each plot represents the best fit to a binding model with two enzyme–ligand complexes (E^-LH^+, E^-L) which is represented by eq 12. For each fit the ligand pK_a was fixed to the independently-determined value.

more, for 5-methyl-8-isobutyl-*N*5-deazapterin the neutral ligand binds ~100 times more strongly to the enzyme in the ternary complex than in the binary complex. Also 7-methyl-8-propyl-*N*5-deazapterin shows quite strong binding in the neutral form compared with 8-propyl-*N*5-deazapterin. The relatively strong binding of these compounds in the neutral form and the generally stronger binding of the neutral forms of all ligands in the ternary complex may be attributable to a hydrophobic interaction between the 8-substituent and the NADPH cofactor in the cofactor site. Molecular graphics modeling studies (unpublished results) using X-ray structures for DHFR complexes[15] suggest that the 8-substituent can be accommodated underneath the

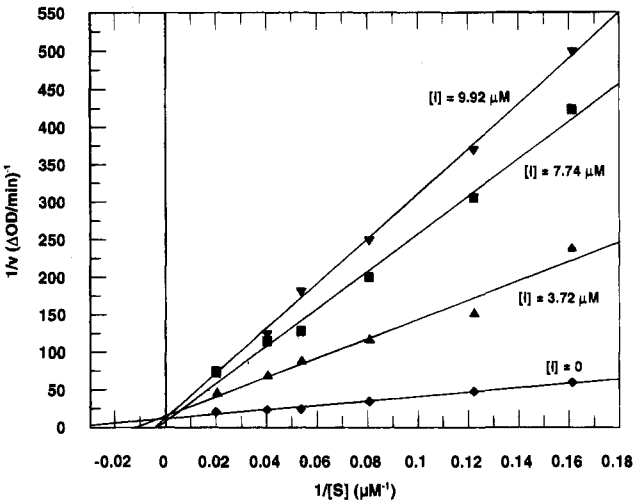


Figure 5. Double reciprocal plot for K_i determination for 6,8-dimethyl-*N*5-deazapterin (**5**) as inhibitor with 6,8-dimethylpterin as variable substrate. The inhibitor concentrations used (0–9.92 μM) are as shown on the graph.

NADPH cofactor. The strong binding of **10** and **15** in the neutral form may arise from the 7-methyl and 8-isobutyl groups, respectively, displacing water from the region of the cofactor. For binding of the protonated ligand to protonated enzyme ($K_{d(EHLH)}$) the values show a smaller variation than for $K_{d(EL)}$ values ranging from 29 ± 16 μM for the 8-propyl compound to 3.7 ± 0.9 μM for 7-methyl-8-propyl-*N*5-deazapterin. While interaction between protonated enzyme and protonated ligand was not observed for the ternary complexes, this finding does not necessarily indicate that this interaction does not exist for these complexes as the lower pH ranges are not experimentally accessible for ternary complexes due to the instability of NADPH in acid conditions (*cf.* Figures 3 and 4).

Binding of 6-Methyl-*N*5-deazapterin. The original design hypothesis for the 8-alkyl-*N*5-deazapterins involved using the greater basicity of these compounds to shift the pH of optimal binding activity into the physiological pH range. Further confirmation that this was indeed the case was obtained by measuring the $K_{d(app)}$ values for 6-methyl-*N*5-deazapterin (**17**) in binary complex with human DHFR. Values of 63 ± 4 and 15.8 ± 0.5 μM were measured at pH 5.4 and 4.3, respectively. These results suggest optimal activity for this compound at pH 4.3 or below which is consistent with its pK_a (4.32 ± 0.06[8]). Comparison with $K_{d(app)}$ data for the binary complex of 6,8-dimethyl-*N*5-deazapterin (**5**) suggests that introducing the 8-methyl group weakens binding of the ligand in the relevant enzyme complexes but only by a factor of ~2.

Type of Inhibition for 6,8-Dimethyl-*N*5-deazapterin. To examine the type of inhibition exhibited by 6,8-dimethyl-*N*5-deazapterin a series of standard kinetic assays to determine the K_i value were performed for chicken DHFR with 6,8-dimethylpterin as the variable substrate. The very low K_m value for dihydrofolate and the very low activity of folate in these pH ranges[2] precludes the use of the natural substrates for this spectrophotometric assay. Figure 5 shows the double-reciprocal plot obtained and suggests that the inhibition is predominantly competitive. This assessment is confirmed by nonlinear regression of the data for which the best fit is obtained for competitive inhibition with a K_i value of 0.80 ± 0.06 μM. Taking into account that 6,8-dimethylpterin has been shown to be a good substrate of DHFR with K_m values in the low micromolar range for reduction to 6,8-dimethyl-7,8-dihydropterin,[10, 11] these inhibition results suggest the 8-alkyl-*N*5-deazapterins bind to DHFR in the substrate site of the enzyme.

Stoichiometry of Binding of 8-Alkyl-*N*5-deazapterins. Binding stoichiometry values for 8-propyl-*N*5-deazapterin (**3**) in binary and ternary complexes with human DHFR were measured as 2.14 ± 0.40 and 1.04 ± 0.22, respectively. The values for binary complexes of 5-methyl-8-propyl- (**12**), 7-methyl-8-propyl- (**10**), 5-methyl-8-isobutyl- (**15**), and 8-benzyl-*N*5-deazapterins (**14**) were 2.18 ± 0.55, 1.08 ± 0.17, 1.30 ± 0.25, and 1.02 ± 0.08, respectively, with one binding site in the ternary complexes also being found for **10** and **15**. These data imply that for ligands with 8-substituents small in size two molecules of ligand can bind to one molecule of enzyme in the absence of cofactor, but that in the ternary complex only one molecule of ligand binds to the enzyme. This suggests that in the binary complex the second molecule of ligand is binding in the cofactor site. For 8-benzyl- (**14**) and 5-methyl-8-isobutyl-*N*5-deazapterins (**15**) only one molecule of ligand binds to the enzyme in both the ternary and binary complexes suggesting that for ligands with larger 8-alkyl substituents the substituent is accommodated in the region of the cofactor binding site in a way which precludes the binding a second ligand molecule, even in the absence of cofactor. The loss of the second binding site for 7-methyl-8-propyl-*N*5-deazapterin can be attributed to the 7-methyl group protruding into the cofactor binding site. This is consistent with the binding data showing reduced cooperativity (see Table 1) with both enzymes compared with the 8-propyl analogue and is also consistent with the proposed mode of binding of the deazapterin ring, i.e., substrate-like.

Summary

The 8-alkyl-*N*5-deazapterins have been shown to be good inhibitors of dihydrofolate reductase (DHFR) with thermodynamic dissociation constants in the micromolar to submicromolar range. The strength of binding of these ligands varies with substitution pattern, enzyme source and complex type (binary or ternary). Straightforward analysis of structure–activity relationships lead to design of a ligand (5-methyl-8-isobutyl-*N*5-deazapterin (**15**); K_d 80 nM) with binding in the ternary complex with human DHFR more than 2 orders of magnitude stronger than for the initial lead compound (8-methyl-*N*5-deazapterin (**1**); K_d 21 μM). Although the binding of **15** is still relatively weak in comparison with known tight binding inhibitors of DHFR such as methotrexate (K_d subnanomolar for vertebrate DHFRs[2]), the relatively simple modifications of the core 8-alkyl-*N*5-deazapterin structure reported in this work and the potential pharmacological advantages such a simple molecule has over an inhibitor such as MTX in terms of its chemical and physical properties, are encouraging and suggest this class of compound has potential for development of novel chemotherapeutic agents. Ongoing work for further derivatives is also investigating their transport properties into cells and cell cytotoxicity (unpublished results) so as to enable compound optimization with respect to a number of

desirable properties, not merely tightness of binding to the enzyme. Also, computer-aided studies involving docking and calculation of relative binding free energies along the lines reported in ref 21 are continuing.

Generally ligand binding was found to be increased substantially by the presence of cofactor with the degree of cooperativity varying with enzyme source and substituent pattern. Studies of the pH dependence of binding have shown it to be due predominantly to interaction between protonated ligand and deprotonated enzyme, but with binding observed also for neutral ligand to deprotonated enzyme which was strongest in the ternary complex. In binary complex, binding of protonated enzyme to protonated ligand was also observed. Identification of 6,8-dimethyl-*N*5-deazapterin as a competitive inhibitor of the chicken DHFR-catalyzed reduction of 6,8-dimethylpterin suggests that these ligands both bind in the substrate site of DHFR. Furthermore, the findings that two molecules of 8-propyl-*N*5-deazapterin bind to human DHFR in the binary complex but only one binds in the ternary complex, and that for ligands with a larger 8-substituent only one molecule binds to DHFR in both the binary and ternary complex, suggest that the 8-substituent is being accommodated in the vicinity of the cofactor site.

Experimental Section

Reagents and Instrumentation. Perkin-Elmer LS-50 and Shimadzu UV 160 spectrometers were used for fluorimetric titration of binding and UV/vis spectrophotometric kinetic studies, respectively. 8-Alkyl-*N*5-deazapterins and *N*5-deazapterins were prepared and characterised previously[8] with assay concentrations determined spectrophotometrically using the previously determined extinction coefficients.[8] Ellis and Morrison[31] MES/TRIZMA/NaCl/ethanolamine pH buffers with $I = 0.2-0.15$ M were used for all binding and kinetic studies. Purified recombinant human DHFR was a gift from Prof. J. H. Freisheim; the samples used contained some denatured material. The chicken liver DHFR from Sigma contained other protein fragments. Active enzyme concentration was determined by methotrexate titration.[24]

Equilibrium Dissociation Constants (K_d). K_d's were determined by fluorimetric titration by a modification of reported procedures.[17] The enzyme fluorescence was excited at 280 nm and the emission intensity measured at 320 nm. The very strong fluorescence of ligands at ~420 nm when excited at their absorption maximum of ~340–360 nm precluded determinations for the ternary complexes from monitoring NADPH fluorescence at ~435 nm.[19] Assays were performed at room temperature (23 °C) in 5 mm cells with a total assay volume of 0.3 mL. Enzyme concentrations in the range 0.14–2.1 μM were used with the higher enzyme concentrations being used for studying the stoichiometry of ligand binding. For determination of dissociation constants for the ternary complexes, NADPH was added in a molar excess of 10:1 compared with the enzyme concentration as suggested by ref 25. A typical assay involved measuring the initial fluorescence of the enzyme/buffer (binary complex) or enzyme/cofactor/buffer (ternary complex) solution and then serially adding 2 μL of stock ligand solution and allowing the mixture to stand for 1 minute before measuring the enzyme fluorescence. Except for the initial fluorescence reading in the human enzyme binary complex determinations at higher pHs, no time dependence for enzyme quenching was observed and stable fluorescence was achieved after 1 min. For the determinations in the human enzyme binary complex at higher pHs, the initial reading was averaged from a small series taken at ~1 s intervals, but after one addition of ligand the fluorescence stabilized as usual over 1 min. Generally, 15 additions were made with the ligand solution concentration chosen so as to give ligand concentrations ~10 times the ligand K_d at the end of the titration. To correct for decrease in the enzyme emission due to absorption of the exciting radiation by the added ligand a tryptophan solution of the same initial emission intensity as the enzyme was titrated with the ligand solution used in the assay. These data were then used to calculate correction factors for the enzyme titration data as outlined by Birdsall *et al.*[17] The corrected enzyme titration data were then fitted using the Grafit nonlinear regression software[32] to eq 3. Quoted values are for single determinations, but analysis of replicate determinations for selected compounds in binary and ternary complexes with both enzymes indicated reproducibility from 15% (1–100 μM) to 20–25% (<1 and >100 μM).

This fit gives estimates of both the K_d and the enzyme fluorescence at saturating ligand concentration (F_s). The value of F_s was then used to estimate the molar fraction (α) of the enzyme bound to ligand after each ligand addition. These values were then used in a plot of L_o/α *versus* $1/(1-\alpha)$[33] where L_o is the ligand concentration in the cuvette after each addition. This results in a linear plot with the slope equal to K_d and the intercept equal to nE_o where n is the stoichiometry of binding. The quoted values are for single determinations but test replicate determinations showed high reproducibility. The pH dependence of binding for a number of ligands for both the binary and ternary complexes was examined by determining the K_d values at a range of pH's (binary complexes 4.15–9.5, ternary complexes 5.2–9.5).

The dissociation constant for the binding of 5-methyl-8-isobutyl-*N*5-deazapterin in the ternary complex with human DHFR with NADPH as cofactor was determined also by monitoring the effect on ligand fluorescence of enzyme binding. This was desirable as practical difficulties with fluorescence signal intensity for the low enzyme concentrations required (~3 times the K_d value) limit the precision with which low K_d values can be measured using enzyme fluorescence quenching. The 8-alkyl-*N*5-deazapterins show intense fluorescence[19] ($\Phi > 0.7$) with emission maxima ~400–420 nm (excitation at 280 and 350 nm). In the presence of enzyme and cofactor the ligand fluorescence is quenched and this effect was used to measure the K_d. Initially a calibration graph was constructed by measuring ligand fluorescence (excitation 280 nm, emission 400 nm) after serial additions of ligand. This experiment was then repeated in the presence of enzyme (0.13 μM) and NADPH (1.5 μM). The data for each experiment were then corrected for dilution effects and the blank emission subtracted. The data for the experiment with enzyme present were then subtracted from the calibration data, and the differences were fitted to eq 3 to obtain the K_d value. The value obtained was in agreement with the rough value determined from the enzyme quenching method.

Kinetic Inhibition Constant (K_i). The kinetic inhibition constant for the binding of 6,8-dimethyl-*N*5-deazapterin (**5**) was determined using 6,8-dimethylpterin[10] as the variable substrate and NADPH at saturating (60 μM) concentration. The assays were performed as previously described[20] using a spectrophotometric assay monitoring the decrease in absorbance of the assay mixture at either 340 nm (NADPH maximum) or 410 nm (6,8-dimethylpterin absorbance only). The assays were performed for a range of inhibitor concentrations and the collected rate data fitted to the standard inhibition equations using Grafit nonlinear regression software.[32]

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