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THERMODYNAMIC STUDY OF THE INTERACTION OF METHOTREXATE, ITS METABOLITES, AND NEW ANTIFOLATES WITH THYMIDYLATE SYNTHASE: INFLUENCE OF FdUMP

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Abstract—A microcalorimetric method was used for the direct study of the interaction of methotrexate, its metabolites, and new antifolates *N*10-propargyl-5,8-dideazafolate (CB 3717) and 2-methyl,2-desamino *N*10-propargyl-5,8-dideazafolate (CB 3819), with thymidylate synthase. We show that 7-hydroxymethotrexate and dideazafolates require the prior binding of dUMP or its fluorinated derivative FdUMP to bind to thymidylate synthase, as does methotrexate. Conversely, we show that methotrexate-G2 can interact directly with the enzyme alone. On the other hand, both dUMP and FdUMP exhibited a large cooperative effect on the affinity for thymidylate synthase of the inhibitors, and surprisingly, no significant difference was shown at this level between the natural substrate dUMP and its fluorinated derivative. It was demonstrated that this cooperative effect had an enthalpic origin. In the presence of FdUMP or dUMP, all the studied compounds except 7-hydroxymethotrexate exhibited a large negative enthalpy variation when binding to thymidylate synthase (from −44 to −91 kJ/mol). CB 3717 and methotrexate-G2 are competitors for the same protein binding site. Polyglutamation of methotrexate lead to compounds with higher affinity (association constants were $6.6 \times 10^3\ M^{-1}$ and $2.3 \times 10^6\ M^{-1}$ for methotrexate and methotrexate-G2 respectively) while hydroxylation has an unfavourable effect (association constant of 7-hydroxymethotrexate inferior to $500\ M^{-1}$). Evidence for the influence of polyglutamation was also provided by the relatively low affinity of dideazofolates for thymidylate synthase (association constant equal to 1.4 and $1.7 \times 10^7\ M^{-1}$ for CB 3717 and CB 3819, respectively), whereas these compounds are known to be strong inhibitors of the enzyme in cells in their polyglutamated forms.

Methotrexate is one of the most widely used anticancer agents, which acts by a tight-binding inhibition of dihydrofolate reductase [1]. Methotrexate† undergoes two metabolic pathways: an intracellular polyglutamation leading to polyglutamyl derivatives, which are also potent inhibitors of dihydrofolate reductase [2], and a hepatic hydroxylation leading to the major plasma metabolite of methotrexate, 7-hydroxymethotrexate, whose affinity for dihydrofolate reductase is lower than that of the parent drug [3].

Dihydrofolate reductase is the main molecular target of methotrexate, but this drug can also inhibit thymidylate synthase by means of its polyglutamyl metabolites [4]. Thymidylate synthase is responsible for the *de novo* synthesis of thymidylate which is essential for DNA biosynthesis: it is the target of 5-fluorouracil, an antineoplastic agent frequently used in combination with methotrexate in anticancer chemotherapy. Indeed, the substrate analogue 5-fluoro-2′-deoxyuridylate (FdUMP) covalently binds to thymidylate synthase and thus blocks its action [5]. Thymidylate synthase is also inhibited by new folate analogues such as *N*10-propargyl-5,8-dideazafolate (CB 3717), or 2-methyl,2-desamino *N*10-propargyl-5,8-dideazafolate (CB 3819) [6].

Allegra *et al.* [4] have studied by enzymatic measurements the inhibition of thymidylate synthase by methotrexate and its polyglutamyl derivatives, and showed that the parent drug acts as an uncompetitive inhibitor of the enzyme, whereas the polyglutamates are non-competitive inhibitors of thymidylate synthase and exhibit a greater inhibiting potency than methotrexate itself.

The effect of folates on nucleotides binding to thymidylate synthase has been extensively studied and an enhancement of the binding by folate-polyglutamyl derivatives has been shown [7]. It has also been found that methotrexate increases the affinity of thymidylate synthase for dUMP [8], and that the drug does not seem to bind in the absence of substrate or substrate analogues [9].

The purpose of our study was directly to examine the binding of methotrexate, its polyglutamyl metabolites, and 7-hydroxymethotrexate to thymidylate synthase by the use of a microcalorimetric method, in order to elucidate the role of metabolism in methotrexate pharmacological activity. Moreover, we wanted to investigate the influence of FdUMP on the formation of the complexes. Finally, we wanted

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† Abbreviations used: methotrexate-G*n*, methotrexate polyglutamates, where *n* represents the number of additional glutamate residues; dUMP, 2′-deoxyuridine 5′-monophosphate; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; CB 3717, N10-propargyl-5,8-dideazafolate; CB 3819, 2-methyl,2-desamino N10-propargyl-5,8-dideazafolate.

to consider the binding to thymidylate synthase of the new antifolate compounds CB 3717 and CB 3819 which are known to be tight binding inhibitors of the enzyme but which are poor inhibitors of dihydrofolate reductase [10].

MATERIALS AND METHODS

Methotrexate was purchased from Specia (Paris, France) and Lederle Laboratories (Wayne, NJ), and dUMP and F-dUMP from the Sigma Chemical Co. (Poole, U.K.). 7-Hydroxymethotrexate was prepared with bovine liver aldehyde oxidase and purified as described in a previous paper [11].

Polyglutamate of methotrexate (methotrexate-G2) was kindly donated by Dr J. Montgomery (Southern Research Institute, Birmingham, AL).

*N*10-Propargyl-5,8-dideazafolate (CB 3717) and 2-methyl,2-desamino *N*10-propargyl-5,8-dideazafolate (CB 3819) were a generous gift of Imperial Chemical Industries Laboratories.

Thymidylate synthase was extracted from a methotrexate-resistant strain of *Lactobacillus casei* [12] and purified according to the procedure described by Lyon *et al.* [13]. Thymidylate synthase was assayed by a spectrophotometric method [14].

Microcalorimetric measurements were performed with a flow calorimeter apparatus (LKB Bioactivity Monitor 2277) at 37° in 0.1 M phosphate buffer, pH 7.40. The phosphate buffer was chosen as an experimental medium to avoid possible interference by proton exchange which may occur during the binding; indeed, phosphate buffer has a very low heat of ionization.

Before each experiment, the baseline was established by pumping a ligand buffer solution into the first circuit of the calorimeter at a flow rate of 20 mL/hr, and pumping the buffer alone into the second circuit at the same flow rate. The sensitivity used was 10 μW full-scale, with a background noise of 0.1 μW. The enzyme (2 to 3×10^{-5} M) was dialysed against the experimental buffer, and 1 or 2 mL was injected into the buffer flow, while the ligand solution was pumped into the second circuit. Whenever necessary, data were corrected from dilution and neutralization heat values.

The experimental heat quantities were corrected in the event of heat of dilution, and were expressed per mole of protein (Q). These measurements were used to determine enthalpy variation (ΔH), association constant (K_a), and consequently free energy variation (ΔG) and entropy variation (ΔS) of protein–drug complexes [15].

The simplest equilibrium complex, with stoichiometry equal to one between a protein (P) and a binding ligand (L) can be written:

$$P + L \rightleftharpoons PL.$$

If P_t and L_t represent the total protein and ligand concentration respectively, and (PL) the complex concentration at equilibrium, the expression of the formation equilibrium constant is:

$$K_a = \frac{(PL)}{(P_t - (PL))\,(L_t - (PL))}. \qquad (1)$$

If L_i is a variable of the total ligand concentration L_t, and (PL_i) the corresponding complex concentration, the experimental heat measurement Q_t (J/L) can be written:

$$Q_i = \Delta H\,(PL_i). \qquad (2)$$

The mathematical expression of the theoretical heat saturation curve $Q_i = f(L_i)$, with P_t constant, is obtained from relationships (1) and (2):

$$Q_i = \frac{1/K_a + L_i + P_t - \sqrt{(1/K_a + L_i + Pt)^2 - 4L_iP_t}}{2}\Delta H \qquad (3)$$

The ΔH or K_a can be calculated from four or five experimental Q_i values obtained at constant P_t and variable L_i (with an L_i range of P_t to $10P_t$). The K_a, ΔH, and if need be stoichiometry values were obtained by using an iterative mathematical treatment of this experimental enthalpic titration curve [15].

Ternary system experiments were performed with a dUMP or FdUMP concentration 10-fold higher than that of thymidylate synthase. If one takes into account the affinity constants of dUMP and FdUMP for *Lactobacillus casei* thymidylate synthase (1.7×10^5 M and 0.95×10^5 M, respectively [16]), and our experimental protein concentration, this excess of dUMP or FdUMP corresponds to a higher than 90% protein saturation.

Results were obtained from three to five separate experiments. They are given with their confidence interval of the mean (P = 0.05).

RESULTS AND DISCUSSION

Table 1 shows the binding heats measured for a large excess of ligand. The inhibitor concentrations were 15-fold higher than that of thymidylate synthase. Given the low affinity of methotrexate and its metabolites for thymidylate synthase, the heat obtained in these conditions does not correspond to protein saturation. Because of the relatively low solubility of these compounds, a maximum concentration ratio of 15 was obtained for experimental thymidylate synthase concentration (about 3×10^{-5} M). Thus, to determine enthalpy variation of the interaction, ΔH, it was necessary to apply an

Table 1. Experimental heat quantities (Q in kJ/mol) observed during the binding of methotrexate, its metabolites and dideazafolates to thymidylate synthase

	Binary system	with FdUMP
Methotrexate	ND	−38 ± 3
Methotrexate-G2	−17 ± 3	−60 ± 2
7-Hydroxymethotrexate	ND	−15 ± 2
CB 3717	ND	−93 ± 3
CB 3819	ND	−72 ± 4

Experiments were performed at 37° in 0.1 M phosphate buffer, pH 7.40, with a ligand to protein concentration ratio of 15.

ND, not detectable.

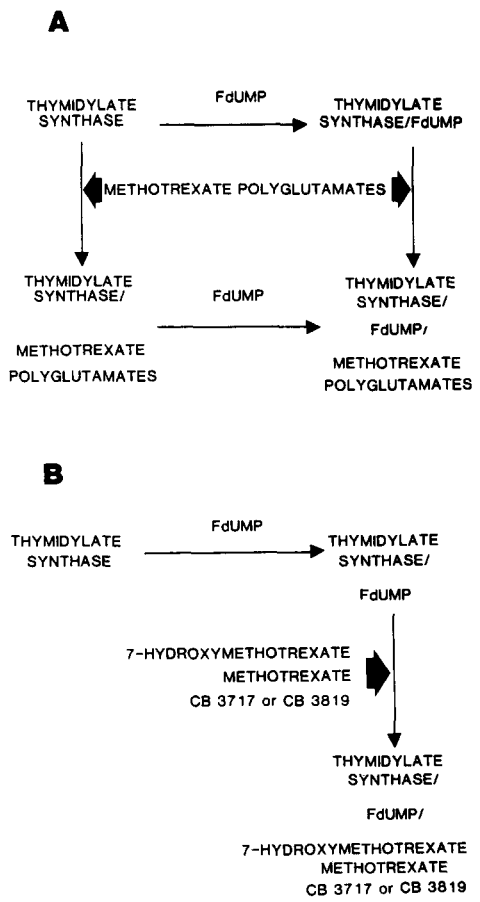

Fig. 1. Mechanism of thymidylate synthase inhibition (A) by methotrexate polyglutamates and (B) by methotrexate, 7-hydroxymethotrexate or dideazafolates.

enthalpic titration method which consists of the measurement of heat of binding of different ligand concentrations to a fixed protein quantity. Moreover, this method allows affinity constants to be determined. Conversely, the affinity of CB 3717 and CB 3819 is so high that binding heats measured in the conditions described above allow direct ΔH determination.

From the binary system results (Table 1, thymidylate synthase–ligand complexes), we can see that only methotrexate polyglutamate binds to the enzyme alone, while methotrexate, 7-hydroxymethotrexate and dideazafolates do not interact directly with thymidylate synthase. These findings are in agreement with those published by Allegra *et al.* [4] concerning the mechanism of inhibition of thymidylate synthase by methotrexate and its polyglutamyl metabolites, which indicated that polyglutamates of methotrexate are non-competitive inhibitors of the enzyme with respect to dUMP, whereas the parent drug acts as an uncompetitive inhibitor. Moreover, our results at 37° corroborate the finding of Galivan *et al.* [8], which show that no binding of methotrexate was detectable at 28°.

We also verified that dideazafolates and methotrexate-G2 compete for the same binding site of thymidylate synthase; indeed, the values of heat of simultaneous binding of the methotrexate metabolite and CB 3717 or CB 3819 was between that of each compound binding alone; this result proves that methotrexate polyglutamate and dideazafolates are competitors for the same protein binding site. Conversely, in the presence of FdUMP, our experimental results show that all the studied compounds exhibit a large exothermic effect when they bind to thymidylate synthase.

Thus, our findings provide direct evidence of the accuracy of the non-competitive inhibition mechanism proposed for methotrexate polyglutamates: these compounds do not require the prior binding of the substrate dUMP or substrate analogues to bind to thymidylate synthase (Fig. 1A). Clearly, non-specific interactions due to the glutamate tail are not involved in the heat exchanged during the binding. Indeed, no heat was observed when methotrexate-G2 interacted with thymidylate synthase previously saturated by CB 3717. On the other hand, our results show that both the hydroxy metabolite and dideazafolates undergo the same binding process as methotrexate: they exhibited an uncompetitive inhibition of the enzyme, i.e. no direct binding of the inhibitor is possible (Fig. 1B).

Surprisingly, no notable difference was observed between dUMP and its fluorinated derivative in their influence on folate binding, as can be seen if we consider enthalpy variations as well as association constants of the inhibitors (Table 2).

Using the enthalpic titration method previously mentioned, we were able to calculate the enthalpy variations and association constants of methotrexate and its metabolites from the heat measurements (Table 2). As mentioned above, only methotrexate polyglutamate interacted in the binary system with thymidylate synthase, and obviously we were able to calculate only the affinity constant of methotrexate-

Table 2. Enthalpy variation (ΔH, in kJ/mol) and association constant (K_a, in M^{-1}) of the thymidylate synthase/antifolate inhibitors complexes

	Binary system		With FdUMP		With dUMP	
	ΔH	K_a	ΔH	K_a	ΔH	K_a
Methotrexate	ND	ND	-45 ± 3	9.1×10^3	-44 ± 3	6.3×10^3
Methotrexate-G2	-23 ± 3	6.6×10^3	-61 ± 2	2.3×10^5	-60 ± 2	1.9×10^5
7-Hydroxymethotrexate	ND	ND	Exothermic	<500	Exothermic	<500
CB 3717	ND	ND	-93 ± 3	1.4×10^7	-91 ± 3	5.0×10^6
CB 3819	ND	ND	-77 ± 4	1.7×10^7	-72 ± 4	6.0×10^6

ND, not detectable.

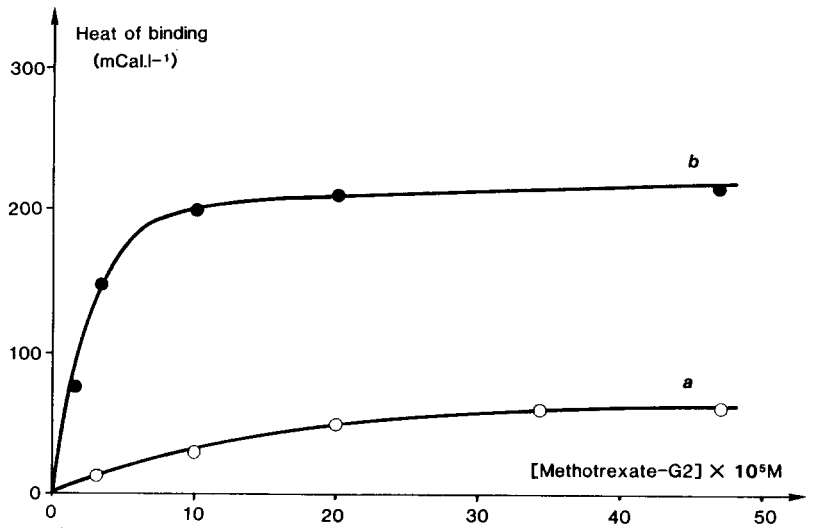

Fig. 2. Fractional saturation heat curves of methotrexate-G2 at 37°, in 0.1 M phosphate buffer pH 7.40: (a) in binary system; (b) in the presence of FdUMP. The binding heats of reaction between various methotrexate-G2 concentrations and a fixed thymidylate synthase quantity (1 mL, 3.0×10^{-5} M) were analyzed according to an enthalpic titration method [13].

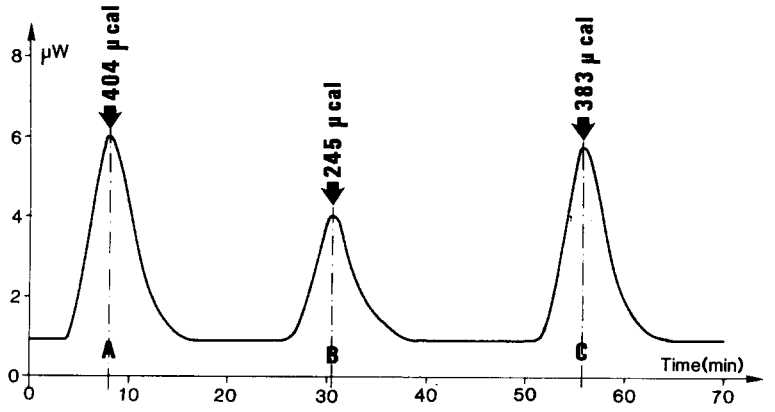

Fig. 3. Microcalorimetric experimental recording of competition between methotrexate-G2 and CB 3717 for thymidylate binding site in the presence of FdUMP. Interaction of 1 mL thymidylate synthase 0.895×10^{-5} M with: (A) CB 3717 0.965×10^{-5} M; (B) methotrexate-G2 1.087×10^{-4} M; (C) CB 3717 0.965×10^{-5} M and methotrexate-G2 1.087×10^{-4} M. Concentrations are the final one in the reaction medium (V = 2 mL).

Table 3. Thermodynamic parameters of the interaction of methotrexate, methotrexate-G2, and dideazafolates with thymidylate synthase

	ΔH (kJ/mol)	K_a (M^{-1})	ΔG (kJ/mol)	ΔS (J/°K/mol)
Binary system				
Methotrexate-G2	−23 ± 3	6.6×10^3	−23 ± 4	0
In the presence of FdUMP				
Methotrexate	−45 ± 3	9.1×10^3	−24 ± 3	−68 ± 6
Methotrexate-G2	−61 ± 2	2.3×10^5	−31 ± 2	−97 ± 7
CB 3717	−93 ± 3	1.4×10^7	−42 ± 3	−165 ± 9
CB 3819	−77 ± 4	1.7×10^7	−43 ± 4	−110 ± 7

G2. On the other hand, we were able to calculate thermodynamic parameters of methotrexate, methotrexate-G2 and 7-hydroxymethotrexate in the presence of FdUMP.

The parent drug exhibited an association constant of $9100\,M^{-1}$ and a ΔH of -45 kJ/mol. The hydroxy metabolite showed a weaker affinity (K_a inferior to $500\,M^{-1}$). Methotrexate-G2 had an association constant of $6600\,M^{-1}$ with the enzyme alone, and about $2 \times 10^5\,M^{-1}$ in the presence of the substrate or of its analogue. Figure 2 represents the fractional saturation heat curves of this compound in the binary system and in the presence of FdUMP. Thus, it can be seen that dUMP or FdUMP are necessary for the binding of methotrexate and 7-hydroxymethotrexate, and that these compounds had a positive cooperative effect on the affinity of methotrexate polyglutamyl derivatives for thymidylate synthase: the affinity is increased by a factor of 30 in the ternary complex enzyme–substrate or analogue–inhibitor. With regards to metabolism, we can conclude that, in contrast with polyglutamation, hydroxylation leads to a compound with a lower affinity for the target enzyme. The same phenomenon had been described in the case of dihydrofolate reductase inhibition by methotrexate and its metabolites [17].

Conversely, given the experimental protein concentration, the high affinity of CB 3717 and CB 3819 was not compatible with direct constant measurements. Thus, to determine their association constant, we used a microcalorimetric method based on the competitive effect of these compounds with methotrexate-G2 at the level of thymidylate synthase binding site [18]. As an example, Fig. 3 is a recording of competition between CB 3717 and the polyglutamyl metabolite of methotrexate in the presence of FdUMP; the comparison of the heat exchange observed when the two compounds bind simultaneously with the enthalpy variation of each of them, allowed K_a ratio determination. In this example, we obtained a K_a ratio of 60 in favor of CB 3717, and the above measured methotrexate-G2 association constant led to a value of $1.4 \times 10^7\,M^{-1}$ for CB 3717 association constant.

From ΔH and K_a values determined above, we calculated the free energy variation (ΔG) and the entropy variation (ΔS) of the binding of methotrexate, methotrexate-G2 and dideazafolates. Results are presented in Table 3. In the binary system, methotrexate-G2 binds to thymidylate reductase with a favourable ΔH. In the presence of FdUMP, the large negative unfavorable ΔS for all compounds, indicates an increase in the orderliness of the system during complex formation. For methotrexate and its polyglutamyl derivative as well as for dideazafolates, the binding is only enthalpically driven. Moreover, it can be seen that the cooperative effect of FdUMP has an enthalpic origin. Indeed, the difference in ΔS observed for methotrexate-G2 between the binary and the ternary complexes (-97 J/°K/mol) is unfavorable. Conversely, the difference in ΔH (-16 kJ/mol) is favorable to binding. Thus these large negative enthalpic and entropic variations are consistent with a coulombic interaction between methotrexate or methotrexate-G2 with thymidylate synthase. This effect is higher for the polyglutamyl metabolite because the glutamate tail adds a charged hydrophilic component to the methotrexate molecule.

Moreover, we are investigating the interaction of trimetrexate with thymidylate synthase. This drug is also an antifolate but, as opposed to methotrexate, it presents, like CB 3717, a lipophilic character due to the absence of the glutamate chain. Preliminary results show that this compound does not interact with thymidylate synthase in a binary system, and that in the presence of FdUMP, trimetrexate exhibits a weaker ΔH than that of methotrexate. This result is further evidence of the importance of the glutamate residue in the binding of antifolate compounds to thymidylate synthase. Thus, this study supports both the lack of interaction of methotrexate in the absence of nucleotides or nucleotide analogues, and the large cooperative effect of dUMP and FdUMP. When methotrexate and 5-fluorouracil are associated, the double inhibition of thymidylate synthase by polyglutamates and FdUMP may have an influence on the kinetics of FdUMP dissociation, as observed with other folate analogues. The great influence of polyglutamation, which allows electrostatic binding of the glutamic chain on thymidylate synthase, has been shown. Conversely, because of the enthalpic origin of the complex formation, methotrexate hydroxylation has an unfavorable effect. This is expected because of the lack of charges on the pteridin moiety of the molecule.

Lipophilic antifolates such as CB 3717 or CB 3819 are known strongly to inhibit thymidylate synthase in cells by means of their polyglutamated forms [19]. The relatively low affinity constant that we measured for these compounds is further evidence of the influence of the polyglutamation process in thymidylate synthase inhibition. It would be of interest to study the binding of dideazafolate polyglutamates if these compounds are sufficiently available.

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