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Effects of antifolates on the binding of 5-fluoro-2′-deoxyuridine monophosphate to thymidylate synthase

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Abstract

Folate based inhibitors of thymidylate synthase (TS) might facilitate binding of 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) to TS similar to the natural reduced folate 5,10-methylenetetrahydrofolate (CH_2-H_4-folate). We studied the lipophilic, non-polyglutamatable antifolates Nolatrexed (NTX) and AG331 and antifolates, that can have a polyglutamate side chain like the natural folate CH_2-H_4-folate; GW1843U89, Raltitrexed (RTX) and Multi-targetted antifolate (MTA) and pentaglutamates (RTX-Glu_5 and MTA-Glu_5). The capacity of these compounds to facilitate the binding of [^{3}H]FdUMP to *Lactobacillus casei* TS and an ammoniumsulphate precipitate of human TS was investigated. Only NTX, RTX-Glu_5 and MTA-Glu_5 facilitated FdUMP binding to *L. casei* TS and their dissociation constant K_d (0.2–0.7 μM) was low compared to CH_2-H_4-folate (2.0 μM). The small lipophilic molecule NTX was favorable to the larger AG331. Polyglutamylation, as indicated by the difference in effect of RTX vs. RTX-Glu_5 and MTA vs. MTA-Glu_5, seems to be important for a classical antifolate to facilitate binding of FdUMP to bacterial TS. Effects of antifolates on FdUMP binding to human TS were different. At a low concentration (0.05 μM) NTX, RTX-Glu_5 and MTA-Glu_5 facilitated 3–5 times higher binding of [^{3}H]FdUMP to TS than CH_2-H_4-folate. At higher concentrations (0.3–5 μM) of NTX, RTX-Glu_5 and MTA-Glu_5 the FdUMP binding decreased. The complex remained stable in the absence of (anti)folate for at least 24 hr. The K_d values of the antifolates for human TS varied from 19 to 387 nM, while the K_d of CH_2-H_4-folate for human TS was 351 nM. The Hill coefficients, which indicated the type of cooperativity of the antifolates in the binding of FdUMP to TS were positive (0.58–0.99) at low concentrations (<0.3 μM) and negative (−0.35 to −0.81) at concentrations >0.3 μM except for GW1843U89, which only showed negative cooperativity (−1.70). It was shown with [^{14}C]NTX that when the binding of FdUMP decreased at high NTX concentrations, the binding of NTX to TS still increased. This also held for the natural substrate dUMP. The negative cooperativity of the antifolates was clearly concentration dependent. The difference between human and *L. casei* TS in the FdUMP binding assays with antifolates can possibly be explained by interaction of the two subunits of human TS, which was absent in *L. casei* TS. The binding of antifolates to one of the two subunits induced a conformational change of the other subunit. This change no longer allowed the binding of FdUMP or dUMP at the active site. In conclusion this study showed that antifolates enhanced the binding of FdUMP to TS, especially at low antifolate concentrations, that are also clinically achievable, e.g. in human plasma.

Keywords: Thymidylate synthase; FdUMP binding; Polyglutamylation; Antifolate; Raltitrexed; ALIMTA

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Abbreviations: TS, thymidylate synthase, EC 2.1.1.45; dUMP, 2′-deoxyuridine-5′-monophosphate; FdUMP, 5-fluoro-dUMP; dTMP, 2′-deoxythymidine-5′-monophosphate; CH_2-H_4-folate, N^5,N^{10}-methylene-5,6,7,8-tetrahydrofolate; RTX, Raltitrexed, *N*-[5-(*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-yl-methyl)-amino)-2-thenyl]-L-glutamic acid (Tomudex, ZD1694); MTA, Multi-targetted antifolate, *N*-(4-(2-(2-amino-4,7-dihydro-4-oxo-3H-pyrrolo[2,3-D]pyrimidin-5-yl)-ethyl)-benzoyl)]-L-glutamic acid (LY231514, ALIMTA, Pemetrexed); GW1843U89, (*S*)-2-(5-(((1,2-dihydro-3-methyl-1-oxobenzo(f)quinazolin-9-yl)-methyl)-amino)-1-oxo-2-isoindolinyl)-glutaric acid; NTX, Nolatrexed, 3,4-dihydro-2-amino-6-methyl-4-oxo-5-(4-pyridylthio)-quinazoline (AG337, Thymitaq); AG331, N^6-[4-(morpholinosulfonyl)benzyl]-N^6-methyl-2,6-diaminobenz-[c,d]-indole glucuronate, RTX-Glu_5, pentaglutamate of RTX; MTA-Glu_5, pentaglutamate of MTA; *L. casei*, *Lactobacillus casei*; K_i, inhibition constant; K_d, dissociation constant.

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1. Introduction

Thymidylate synthase (TS) is a key enzyme in the *de novo* pyrimidine nucleotide synthesis. It catalyses the methylation of dUMP by the reduced folate CH_2-H_4-folate into dTMP [1]. The enzyme consists of two identical dimers, with one active site on each subunit. An ordered sequence of addition of substrates takes place during catalysis. First dUMP binds at the active site; the pyrimidine and ribose moieties provide a docking surface for the binding of the second substrate CH_2-H_4-folate. The whole process, including product formation and release of the products H_2-folate and dTMP induces large conformational changes of the enzyme [2]. The study by Dev *et al.* [3] proposed that the substrate binding sites of human TS exhibit ligand-induced negative cooperativity. This was based on the observation that only 0.6 mol of [^{14}C]CH_2-H_4-folate was bound per mol of dimeric enzyme in a ternary complex with dUMP, rather than the 2 mol expected if both subunits were available for binding. The interaction of the active sites of TS has also been described by Aull *et al.* [4] who suggested that the two binding sites of *Lactobacillus casei* TS are non-equivalent because FdUMP decayed more rapidly from one site than the other when in a ternary complex with CH_2-H_4-folate. Also the two active sites of *L. casei* TS bind dUMP with different affinities [2]. Other supporting data on the interaction of the active sites was obtained with X-ray crystallography, showing that each substrate binding site of TS is composed of amino acid residues from both subunits [2]. The two binding sites influence enzyme kinetic parameters such as the Hill coefficient.

The biological important feature of TS is its essential and unique function in DNA synthesis, because it catalyses the *de novo* synthesis of thymidine nucleotides. This property has made the enzyme an attractive target for cancer chemotherapy. Analogues of either substrate have been developed as potential inhibitors of TS. The pyrimidine analogue FdUMP is a potent inhibitor of TS and it has a K_i of 1 nM [5]. FdUMP forms a stable ternary complex with the enzyme and CH_2-H_4-folate [5]. This binding differs from that of the normal substrate dUMP, because it leads to an irreversible dead end complex, resulting in kinetic properties, which formed the basis for this study. The anticancer drugs that can provide FdUMP in a cell are fluoropyrimidines such as 5-fluorouracil (5FU) or 5-fluoro-2′-deoxyuridine.

Alternatively, analogues of CH_2-H_4-folate have been developed to inhibit TS at the other binding site. Most of them act as non-competitive (e.g. GW1843U89) [3] or mixed non-competitive analogues (e.g. RTX) [6] of CH_2-H_4-folate. All these compounds also serve as anticancer agents. Two different approaches have been used in the development of these compounds. Firstly, structural analogues of folic acid were designed, so called classical antifolates. Representatives of this group are RTX [6], MTA [7] and GW1843U89 [8]. These compounds enter the cell *via* one of the folate transport systems and are retained in the cell after polyglutamylation. All compounds form good substrates for folylpolyglutamate synthetase and one (GW1843U89) to five (RTX, MTA) glutamate chains can be linked to the parent antifolate molecule. The affinity for TS is increased by polyglutamylation [6,7]. Secondly, non-classical antifolates were designed. Knowledge about the crystal structure of TS, initiated the development of antifolates such as AG331 [9] and NTX [10] with a lipophilic structure that lacks a glutamate moiety. This allows passive diffusion over the cell membrane and compounds are not polyglutamated but still have a high affinity for TS. K_is are 2 and 11 nM for AG331 and NTX, respectively.

In a previous study, based on earlier studies of other groups [11] we showed that in a cell free system using *L. casei* TS, naturally occurring (reduced) folates, e.g. tetrahydrofolate and 5-methyltetrahydrofolate, could also facilitate FdUMP binding to TS, albeit to a lesser extent than the natural cosubstrate CH_2-H_4-folate [12]. Our hypothesis was that the described antifolates might show a similar effect, based on a report on FdUMP and other quinazoline folate analogues [13] and a study that described that GW1843U89 enhanced FdUMP binding to *E. coli* TS [14]. This characteristic could be of interest for both preclinical and clinical studies combining 5FU treatment with antifolates.

Antifolate dependent FdUMP binding to TS was evaluated in a cell free system using purified *L. casei* TS and [6-^{3}H]FdUMP. Additionally we also used human TS partially purified using ammoniumsulphate precipitation. In both systems we tested several concentrations of the different antifolates. Enzyme kinetic parameters were calculated to evaluate the effects of two artificial substrates and their meaning for TS inhibition.

2. Methods

2.1. Materials

Lactobacillus casei TS, purified enzyme was obtained from BIOPURE. The human TS was partly purified by ammoniumsulphate precipitation of TS [5] from a TS overexpressing cell line W1L2:C1 (kindly provided by Dr. A.L. Jackman, Institute of Cancer Research, Sutton, UK). H_4-folate obtained from Sigma Chemical Co. was converted into CH_2-H_4-folate by addition of formaldehyde [15]. RTX was a gift from AstraZeneca Pharmaceuticals. The pentaglutamate RTX-Glu_5 was kindly donated by Dr. A.L. Jackman. MTA and its pentaglutamate LY247292 (referred to as MTA-Glu_5) were generously supplied by Eli Lilly. AG331, NTX and [^{14}C]-labeled NTX (specific activity 18.2 mCi/mmol) were donated by Agouron Pharmaceuticals. GW1843U89 was a gift from GlaxoWellcome. [6-^{3}H]FdUMP (specific activity 16.6 Ci/mmol was purchased from Moravek biochemicals Inc. [5-^{3}H]dUMP (specific activity 18.6 Ci/mmol) was obtained from Amersham.

2.2. FdUMP ligand binding assay

The FdUMP ligand binding assay was performed as described previously [12]. Final concentrations in the assay of CH_2-H_4-folate and antifolates were varied from 0.01 to 5 μM, the [^{3}H]FdUMP concentration was 0.038 μM (0.13 μCi) and 114 ng protein of the *L. casei* TS stock or 570 ng protein of the human TS precipitate from W1L2:C1 were used. The standard assay time was 15 min at 30°, at longer assay times comparable results were found (data not shown). After this incubation, free and complexed [^{3}H]FdUMP were separated by an acid charcoal wash. The radioactive complex was quantified by liquid scintillation counting. The blank in this assay contained FdUMP and TS, but no (anti)folate, so all values were corrected for the formation of binary complex. Although the preparations were partially purified it is unlikely that other FdUMP binding proteins were present considering the very low blanks. The preparations were dialyzed making a different ion concentration between the formulations unlikely. Only the protein content was different, but for CH_2-H_4-folate the used concentrations were in a linear range.

In order to explain some of the results obtained with human TS and NTX and the pentaglutamates of RTX and MTA assay, we determined the stability of the ternary complex under assay conditions, when 1 μM (anti)folate was used in the assay. After the acid charcoal wash following 15 min incubation, the supernatant was again incubated at 30° for 15 min, 30 min or 20 hr and washed again with acid charcoal to remove [^{3}H]FdUMP, which was dissociated from the complex. We also used a very high antifolate concentration (250 μM) in the assay to see whether antifolates could reduce or prevent the binding of [^{3}H]FdUMP. We also tried to achieve this effect by a preincubation of TS with the peak concentration of RTX-Glu_5 (0.1 μM), GW1843U89 (0.05μM), NTX (0.25 μM) or 0.25 μM CH_2-H_4-folate for 2 hr at 30°, prior to the 15 min assay with [^{3}H]FdUMP.

The availability of [^{14}C]NTX enabled us to perform the assay with two labeled substrates [^{3}H]FdUMP and [^{14}C]NTX. After the enzyme assay and charcoal wash, supernatants were measured for both ^{3}H and ^{14}C radioactivity. Finally, [^{3}H]dUMP (25 μM) was used instead of FdUMP to examine whether the binding of this substrate to TS was also influenced by NTX.

The interaction of the ligand in the binding assay at enzyme kinetic level [16] was analyzed as the dissociation constant K_d and the Hill coefficient.

3. Results and discussion

Binding of [^{3}H]FdUMP to *L. casei* TS was facilitated by the natural cosubstrate CH_2-H_4-folate and some of the antifolates (Fig. 1). A concentration dependent increase of FdUMP binding was observed until a plateau was reached (not shown). The highest binding was obtained with the natural cosubstrate, but also the pentaglutamates of RTX and MTA could facilitate FdUMP binding. The monoglutamates RTX, MTA and GW1843U89 were inactive. The lipophilic antifolates showed a differential effect: NTX supported FdUMP binding to *L. casei* TS, whereas AG331 did not. The K_d constants for each of the compounds were determined by Scatchard plots (Table 1, Fig. 1 insert). The Hill coefficients (Table 1) were similar for all compounds and indicate positive cooperative binding of FdUMP and (anti)folates to TS.

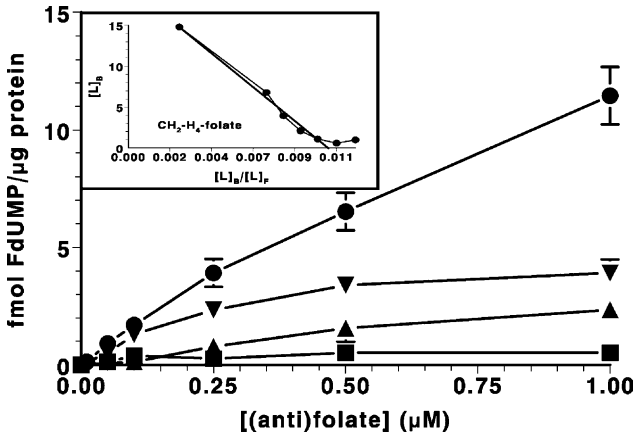

Fig. 1. FdUMP binding to *L. casei* TS facilitated by several concentrations of the (anti)folates: CH_2-H_4-folate (●), RTX-Glu_5 (▼), NTX (▲) and MTA-Glu_5 (■). Indicated variations are the SD of the mean of three separate experiments. The results of the other antifolates (RTX, MTA, AG331 and GW1843U89) were below the detection limit of the assay. Insert: Scatchard plot of FdUMP binding to *L. casei* TS as a function of the CH_2-H_4-folate concentration.

FdUMP binding to human TS could be facilitated by all antifolates (Fig. 2a–c), but there were clear differences between mono- and pentaglutamates. The compounds that showed the highest binding in the assay with bacterial TS (NTX, MTA-Glu_5 and RTX-Glu_5) also turned out to be the most potent ones in the assay with human TS. An important factor that influenced the binding process was polyglutamylation. This can be explained by the higher affinity of polyglutamate forms for TS [6,7]. For the non-polyglutamatable antifolates (AG331 and NTX), the size of the larger AG331 molecule is probably a disadvantage in

Table 1
Parameters of the enzyme kinetic analysis of FdUMP and (anti)folate binding to bacterial TS

(Anti)folate	K_d (μM)	Hill coefficient
CH_2-H_4-folate	2.00 ± 0.7	1.0 ± 0.05
RTX-Glu_5	0.23 ± 0.6	1.0 ± 0.1
MTA-Glu_5	0.43 ± 0.4	1.6 ± 0.6
NTX	0.74 ± 0.3	1.4 ± 0.4

The dissociation constant K_d and the Hill coefficient are means ± SD of three separate experiments. K_d values for RTX, MTA, AG331 and GW1843U89 were not evaluable because of negative binding values. Values are not significantly different.

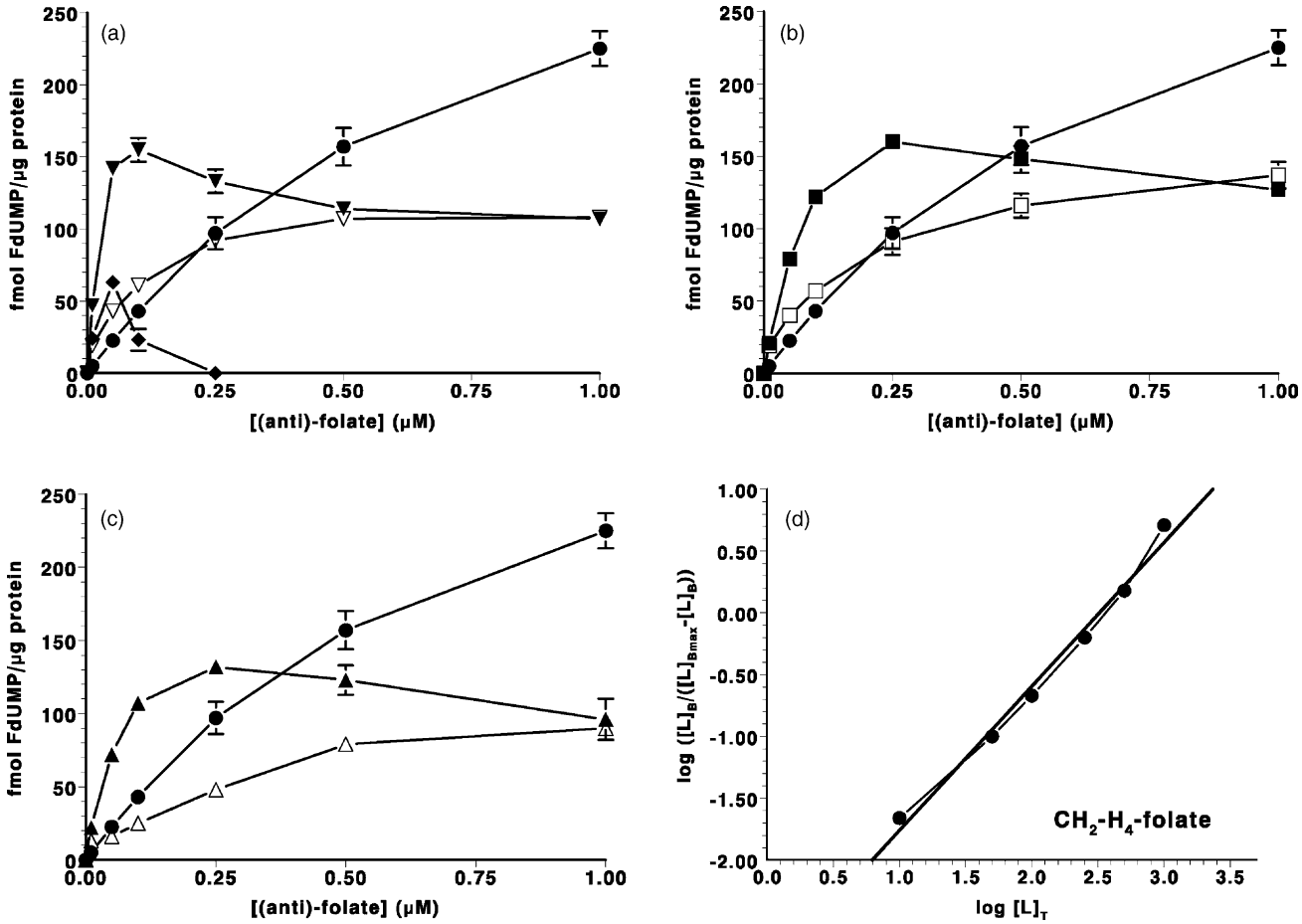

Fig. 2. (a–c) Comparison of the capacity of CH_2-H_4-folate and different antifolates to facilitate FdUMP binding to human TS. CH_2-H_4-folate (●), RTX (▽), RTX-Glu_5 (▼), GW1843U89 (◆), NTX (▲), AG331 (Δ), MTA (□) and MTA-Glu_5 (■). Values are means ± SD of three experiments, for some values the SD was very small (within the symbol). (d) Hill plot of FdUMP binding to human TS as a function of the CH_2-H_4-folate concentration (●).

promoting FdUMP binding to TS, as compared to the smaller NTX molecule.

However at concentrations below 0.3 µM, these compounds facilitated a higher extent of FdUMP binding to TS, than the natural cosubstrate. In contrast to bacterial TS, all polyglutamates, GW1843U89 and NTX showed a peak value in FdUMP binding to human TS, resulting in a lower FdUMP binding per pmol TS at 5 µM (not shown) than at 1 µM antifolate. The concentration at which the peak value occurred differed per antifolate. Ternary complex formation of TS, FdUMP and CH_2-H_4-folate was concentration dependent with CH_2-H_4-folate similar to *L. casei* TS. The FdUMP binding with antifolates and human TS, especially at high concentrations, was very different from the results with bacterial TS. The Scatchard analysis (Table 2) showed a high K_d (351 nM) for CH_2-H_4-folate indicating a relatively unstable ternary complex and a low K_d (23 nM) for GW1843U89 referring to better FdUMP-TS-GW1843U89 complex formation. All K_ds of the antifolates were based on the first part of the FdUMP binding curve with low antifolate concentrations. The higher amount of FdUMP binding that was facilitated by polyglutamates of RTX and MTA is in line with their higher affinity for TS compared to the monoglutamates. Keyomarsi and Moran [13] observed that a tightly bound complex is formed between TS, dUMP and the pentaglutamate of 10-propargyl-5,8-dideazafolic acid (CB3717), but not with the monoglutamate. The Hill

Table 2
Parameters of the enzyme kinetic analysis of FdUMP and (anti)folate binding to human TS

(Anti)folate	K_d (nM)	Hill coefficient 1	Hill coefficient 2
CH_2-H_4-folate	351 ± 92	1.24 ± 0.02	
RTX	93 ± 15	0.58 ± 0.02	
RTX-Glu_5	19 ± 4[a,b]	0.83 ± 0.04	−0.35 ± 0.01
MTA	137 ± 12	0.64 ± 0.03	
MTA-Glu_5	75 ± 9[c]	0.99 ± 0.03	−0.67 ± 0.02
NTX	53 ± 10	0.87 ± 0.02	−0.73 ± 0.01
AG331	387 ± 44	0.82 ± 0.05	
GW1843U89	23 ± 2		−1.70 ± 0.30

The dissociation constant K_d and the Hill coefficients 1 and 2 are means ± SD of three experiments. Hill coefficient 1 refers to the curve left of the peak value and positive cooperativity, as illustrated in Fig. 2d. Hill coefficient 2 refers to the right part of the curve after the peak value and negative cooperativity determined by the Hill plot.

[a]Statistics: RTX-Glu_5 vs. MTA-Glu_5, $P = 0.006$.

[b]Statistics: RTX-Glu_5 vs. NTX, $P = 0.005$.

[c]Statistics: MTA-Glu_5 vs. NTX, $P = 0.05$ (Student's *t*-test).

coefficients (Table 2 and Fig. 2d) at low concentrations indicated positive cooperativity, but at higher antifolate concentrations (>0.3 μM) the values were negative, which referred to negative cooperative binding of FdUMP and antifolates to TS. The Hill coefficient is also influenced by the fact that the enzyme has two binding sites and not one as in straight forward enzyme kinetics analysis.

Comparing the results of *L. casei* in human TS we assume that the antifolates have a higher affinity for human TS, since a higher FdUMP binding could be facilitated by all antifolates than observed for *L. casei* TS. The compounds with no activity in the bacterial TS assay (AG331, RTX and MTA) showed a curve with a shape similar to that of the natural cosubstrate. The exception was GW1843U89, which showed no activity in the bacterial assay and a changing activity in the human TS assay, similar to the RTX and MTA polyglutamates and NTX. This could be related to the very high affinity of GW1843U89 for TS and its unique binding to TS. The binding of GW1843U89 to TS induces a distortion in the active site as has been described by Weichsel *et al*. [14]. Furthermore GW1843U89 promotes the binding of dUMP and FdUMP to *E. coli* TS about 2-fold [17]. However this capacity was limited to only one subunit, whereas the concentration GW1843U89 (6–10 μM) at 0.2 μM TS and 0.02–5 μM FdUMP was much higher than ours. Already at 0.25 μM GW1843U89 we observed absence of the FdUMP binding to human TS and the negative values for FdUMP binding at higher concentrations showed that there was also a reduction of binary TS–FdUMP complex. A similar effect could be observed when very high concentrations of NTX and MTA-Glu_5 were used. The binding of FdUMP could be reduced to 0 and even below the level of binary complex formation (negative values).

The stability of a complex of CH_2-H_4-folate or RTX-Glu_5 or NTX, TS and [^{3}H]FdUMP did not differ under the assay conditions. Shortly after formation (15 and 30 min) there was no change in the amount of tritiated complex. After 20 hr incubation at 30° only 12, 7 and 13% of the complex had dissociated when formed in the presence of CH_2-H_4-folate, NTX and RTX-Glu_5, respectively.

A pre-incubation with the peak concentrations of RTX-Glu_5 (0.1 μM), GW1843U89 (0.05 μM) or NTX (0.25 μM) resulted in 56, 37 and 51% reduction of the FdUMP binding to human TS, respectively, when compared to no pre-incubation. However, a similar effect (51% reduction) was observed when we used 0.25 μM CH_2-H_4-folate (data not shown). Keyomarsi and Moran [13] reported a similar effect. After 2 hr pre-incubation of mouse TS isolated from L1210 cells with 0.1 μM the antifolate CB3717-pentaglutamate, still 50% of the FdUMP binding capacity of TS was retained. They proposed a model where CB3717 and dUMP bind to one subunit of TS, inducing a conformational change of the second subunit that then could bind FdUMP. Other studies also showed that with ligands such as dUMP, FdUMP, CH_2-H_4-folate and GW1843U89, binding to TS can result in large conformational changes [2,14,18]. Antifolate binding to the first subunit of TS may induce a conformational change of the second subunit causing dissociation of FdUMP from this subunit. GW1843U89 reduced the binding of FdUMP from 2 to 1 mol/mol of dimer [14]. Now this loss of FdUMP binding has been observed for several antifolates and it could be a more general effect of high concentrations (anti)folate. The Hill coefficient changing from positive to negative will be influenced by the conformational change.

The formation of a ternary complex of TS, FdUMP and antifolate was further investigated with two labeled substrates [^{3}H]FdUMP and [^{14}C]NTX. As in all other assays both substrates were added simultaneously. At low concentrations (<0.3 μM) NTX we observed a concentration dependent increase in the [^{3}H]-labeling of the complex (Fig. 3a). The labeling gradually decreased when the NTX concentration increased. On the contrary the [^{14}C]-labeling increased with rising NTX concentrations and these results showed the start of a sigmoidal shaped curve. When we used the normal substrate [^{3}H]dUMP instead of [^{3}H]FdUMP, again a peak value of [^{3}H]-labeled com-

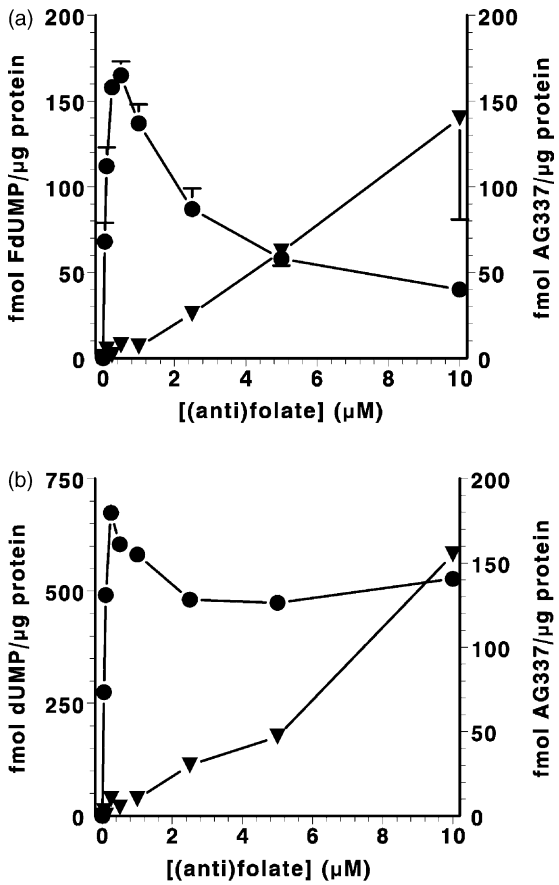

Fig. 3. (a) Complex formation of FdUMP, TS and NTX evaluated by [^{3}H]FdUMP bound to human TS (●) and [^{14}C]NTX bound to TS (▼). (b) Complex formation of dUMP, TS and NTX evaluated by [^{3}H]dUMP bound to TS (●) and [^{14}C]NTX bound to TS (▼). Values are means ± SD of three experiments. Final [^{3}H]FdUMP concentration was 0.038 μM, final [^{3}H]dUMP concentration was 25 μM.

plex was observed at 0.25 μM NTX followed by a gradual decline (Fig. 3b). The results on [^{14}C]-labeled complex obtained with dUMP were comparable to the values obtained with FdUMP. The results on the [^{3}H]-labeled complex showed that dissociation of dUMP from a complex with human TS and NTX was only 25% of the highest binding whereas more than 75% dissociation of FdUMP occurred at a concentration of 5 μM NTX. Results obtained with RTX could give some explanation. Rutenber and Stroud [19] evaluated the structural determinants of the ligand protein interaction of dUMP, RTX and TS, based on the crystal structure of *E. coli* TS. RTX possibly acts as an inhibitor of the multi-step TS reaction by catalyzing the first part of the reaction, the covalent bond formation between substrate dUMP and the catalytic thiol group Cys146. The enzyme is then trapped at a reversible step midway along the reaction pathway. Also for GW1843U89 crystallographic data about complex formation with dUMP and TS have been published [14], but they do not give indications for dUMP dissociation other than that the reaction is reversible. Weichsel *et al.* [14] reported a K_d of 17 nM for GW1843489 binding with FdUMP. We did not evaluate the K_d of FdUMP binding for our enzyme preparation; this value is usually in the low nanomolar range for human TS [20]. The kinetic analysis of FdUMP and dUMP binding to TS as a consequence of the folate cosubstrate and their different analogues support that conformational changes can be suggested by some analogues.

The consequences of our results on the interaction of FdUMP and antifolates obtained in cell free systems for cellular systems and drug combination testing could be the following: antifolates are very potent drugs and the concentrations at which they are active are usually below 0.3 μM (except AG331 and NTX). At these concentrations they can enhance FdUMP binding. A combination of antifolates and 5FU can induce at least additive growth inhibition of (tumor) cells [21,22,23] through a very efficient inhibition of TS [21]. FdUMP facilitates a tight binding of GW1843489 [14]. It has been shown previously that a high intracellular folate pool can abrogate the effect of antifolates [24]; however, in a combination with 5FU this negative effect can be reversed since normal folates also stabilize the ternary complex [12]. Therefore it can be expected that ternary complex formation of FdUMP, TS and either a natural folate or an antifolate, would increase TS inhibition.

Several clinical studies combining antifolates and 5FU have been performed in which sequential administration of the drugs was used [25,26]. We observed decreased FdUMP binding when preincubation with antifolates was performed, which would favor 5FU administration before antifolate administration. Also simultaneous administration could result in optimal FdUMP binding, depending on antifolate dose and pharmacokinetics. These data support future clinical studies combining antifolates with 5FU.

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