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Binding of 5-Fluorodeoxyuridylate to Thymidylate Synthase in Human Colon Adenocarcinoma Xenografts

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Abstract—*The formation and stability of the covalent ternary complex formed between thymidylate synthase (E.C. 2.1.1.45), 5-fluoro 2'-deoxyuridylate (FdUMP) and 5,10-methylenetetrahydrofolate (CH_2-H_4PteGlu) has been examined in cytosols derived from xenografts of human colon adenocarcinomas. The rate of association (k_a) for FdUMP was low being between 3.4 ± 0.9 and 10.2 ± 2.6 × 10^6 M^{-1} min^{-1}, with the lowest k_a value being determined in cytosols from a tumor ($HxELC_2$) which has demonstrated some sensitivity to 5-fluoropyrimidines. Relative to reported k_a values for human leukemic cells, the rate of association of FdUMP was 20- to 59-fold lower. This difference is not a consequence of FdUMP catabolism, or metabolism of CH_2-H_4PteGlu. In cytosols the apparent K_m values for dUMP (3.6–4.2 μM) and [6RS]-CH_2-H_4PteGlu (25–26.7 μM) were similar to reported values for human enzyme. Data derived from cytosols were similar to those derived using affinity purified enzyme from $HxVRC_5$ colon adenocarcinoma xenografts. The net dissociation of [6-^{3}H] FdUMP from the covalent ternary complex was 31–33 min in the absence of added CH_2-H_4PteGlu, and the rate of dissociation was dependent upon the concentration of cofactor. The concentration of [6RS]-CH_2-H_4PteGlu required to stabilize ternary complex derived from $HxELC_2$ cytosols was slightly lower than that required for the same degree of stabilization of complex formed in cytosols from resistant tumors ($HxGC_3$, $HxVRC_5$). Addition of 5-CHO-H_4PteGlu, 5-CH_3-H_4PteGlu, H_2PteGlu, and PteGlu did not stabilize the covalent complex, but H_4PteGlu substituted for CH_2-H_4PteGlu.*

INTRODUCTION

THYMIDYLATE SYNTHASE (E.C.2.1.1.45) catalyzes the reductive methylation of deoxyuridylate to form thymidylate. In mammalian cells this pathway is unique for *de novo* synthesis of dTMP, and hence has been selected as a target for potential anticancer agents [1, 2]. 5-Fluoropyrimidines are metabolized to FdUMP, which in the presence of CH_2-H_4PteGlu forms a quasi irreversible covalent ternary complex [2]. The dissociation of this inhibitory complex is first order and dependent upon the concentration of unbound CH_2-H_4PteGlu [3]. Consequently, the duration of inhibition of thymidylate synthase *in vivo* may be dependent upon the endogenous concentration of CH_2-H_4PteGlu or its polyglutamate forms. The concentration of CH_2-H_4PteGlu required to stabilize the ternary complex appears also to depend upon the rate at which FdUMP associates (k_a) with thymidylate synthase [4]. In the study of Bapat *et al.* [4], a line of CCRF–CEM human leukemia selected for resistance to 5-fluoro 2'-deoxyuridine (FdUrd), demonstrated an altered thymidylate synthase. This enzyme was characterized by a 14-fold decrease in k_a, and required 3–4 times greater concentrations of CH_2-H_4PteGlu to give a similar increase in complex stability compared to the parent line.

In previous studies [5] using human tumor xenografts the formation of covalent ternary complex was examined in cytosols derived from 5-fluorouracil (FUra)-sensitive or intrinsically resistant colon adenocarcinomas. These studies suggested that in preparations from resistant tumors, maximal ternary complex was formed only in the presence of added cofactor (CH_2-H_4PteGlu). Alternatively, it could be argued that complex formed in these preparations was less stable than those formed in cytosols from FUra-sensitive tumors. In the present study we have examined the formation and stability of covalent ternary complex in tissue cytosols. The significance of these data to therapeutic modulation of thymidylate synthase is discussed.

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MATERIALS AND METHODS

Tumor lines

Human colon adenocarcinoma xenografts

$HxVRC_5$, $HxGC_3$ and $HxELC_2$ have been described previously [6, 7]. Briefly, $HxELC_2$ shows some sensitivity to 5-fluoropyrimidines whereas $HxVRC_5$ and $HxGC_3$ are intrinsically resistant [7]. Tumors were passaged bilaterally in the subcutaneous space of mice immune-deprived by thymectomy, cytosine arabinoside and whole body irradiation as previously described [8].

Formation of ternary complex

Excised tumors were homogenized for 30 sec (polytron, Brinkman Instruments) in ice-cold buffer A (60 mM sodium phosphate pH 7.4 containing 0.12% w/v bovine serum albumin and 10 mM β-mercaptoethanol). Routinely, complexes were formed in 100,000 ***g*** (60 min, 4°C) supernatants using 28 nM [6−^{3}H]FdUMP (Moravek Biochemicals, Brea, CA) and 100 μM [6RS]-CH_2-H_4PteGlu, and incubated at 37°C for 120 min. Complexes were separated from unbound FdUMP and folate by gel filtration at 4°C on a G-25 Sephadex column (14 × 2.5 cm) equilibrated in buffer A. For experiments in which the rate of FdUMP association (k_a) was examined, or the affinity of either [6RS]-CH_2-H_4PteGlu or dUMP for thymidylate synthase was determined, G-25 chromatographed cytosols were used.

Kinetic studies

Measurement of the rate of [^{3}H]FdUMP association to thymidylate synthase was essentially as described by Lockshin and Danenberg [9]. In all experiments the concentration of [6RS]-CH_2-H_4PteGlu (100 μM) greatly exceeded that of the ligand binding sites (0.28–16.85 nM). The concentration of [^{3}H]FdUMP was varied between 5 and 100 nM, in a total reaction volume of 1 or 2 ml. Cytosols were routinely chromatographed using G-25 Sephadex, as described above. Reaction velocity was linear over 4 min subsequent to adding prewarmed cytosol (37°C, 5 min preincubation). Reactions were terminated by pipetting aliquots of reaction mixtures (100 μl) into 1.2 ml ice-cold quench solution (2% charcoal, 0.5% albumin and 0.05% dextran) as described [5]. Under these conditions, the apparent bimolecular rate constant (k_a) for [^{3}H]FdUMP association to thymidylate synthase was calculated from the equation [9, 10]

$$k_a = \frac{1}{[E_0]-[FdUMP_0]t} \ln \frac{[FdUMP_0]\,([E_0]-[X])}{[E_0]\,(FdUMP_0]-[X])}$$

where $[E_0]$ is the initial concentration of enzyme binding sites, $[FdUMP_0]$ is the initial concentration of [^{3}H]FdUMP, and [X] is the concentration of ternary complex at time t (min).

Measurement of the catabolism of [^{3}H]FdUMP

Where there is rapid conversion of FdUMP or dUMP to its deoxynucleoside or base, this could significantly influence the results obtained in determination of k_a and K_m values. The rate at which [^{3}H]FdUMP was catabolized was examined in G-25 chromatographed cytosols derived from each tumor line. The final reaction (60 μl) contained 40 μl cytosol, 500 μM [^{3}H]FdUMP (sp. act. 66 mCi/mmol) and buffer A. Reaction mixtures were incubated at 37°C for up to 30 min. At the appropriate time an aliquot (10 μl) was removed and mixed with 3 μl perchloric acid (1 M) and retained on ice for 5 min. After centrifuging 5.3 μl KOH (1 M) were added, and left on ice a further 5 min. The sample was again centrifuged and 10 μl of the supernatant was analyzed by HPLC. Samples were eluted from a Partisil 10/25 SAX column (Whatman, NJ) using a linear gradient from 2.5 to 125 mM $(NH_4)_2PO_4$ at pH 3.5. Both FdUrd and FUra eluted with the void volume (≈ 3.5 min) whereas the retention time for FdUMP was 17.6 min. Radiolabel was determined in 0.5 min fractions. Protein concentration was determined using the BioRad assay (BioRad, Richmond, CA).

Metabolism of $^{14}CH_2$-H_4PteGlu

Conversion of [^{14}C]CH_2-H_4PteGlu was examined in Sephadex G-25-chromatographed cytosols. Radiolabelled CH_2-H_4PteGlu was formed by combining 630 nmol [^{14}C]HCHO (New England Nuclear, sp. act. 2.85 mCi/mmol) and 1.1 μmol [6RS]-H_4PteGlu in buffer A containing a final concentration of 143 mM β-mercaptoethanol and 10 mM sodium ascorbate. The solution was incubated at room temperature for 15 min, and subsequently at 37°C for 30 min. To examine metabolism of $^{14}CH_2$-H_4PteGlu, 350 μl cytosol combined with FdUMP (final conc. 1 μM, to inhibit the thymidylate synthase reaction), 220 μl radiolabelled folate, 143 mM β-mercaptoethanol, 10 mM sodium ascorbate in a final volume of 700 μl. Reactions were incubated at 37°C and were terminated at time 0 and 60 min by placing 350 μl aliquots into a boiling water bath for 3 min. Mixtures were subsequently cooled on ice and centrifuged. Supernatants were mixed with 5-CH_3-H_4PteGlu, and 180–200 μl analyzed by HPLC. Samples were chromatographed on a C-18 column (Advanced Separation Technologies, Inc., Whippany, NJ), using a buffer containing 5 mM tetrabutylammonium phosphate (Waters Associates, Milford, MA) and 25% MeOH. This system has the advantage over that used previously [11] in

that CH_2-H_4PteGlu (R_f = 27.4 min) can be separated from 5-CH_3-H_4PteGlu (R_f = 31.4 min). Radioactivity was determined in 0.5 min (0.5 ml) samples.

Measurement of net dissociation of [^{3}H]FdUMP-labeled ternary complex

Ternary complex was formed using 28 nM [^{3}H]FdUMP, 100 μM [6RS]-CH_2-H_4PteGlu and cytosol, incubated at 37°C for 120 min, and separated from unbound folate and nucleotide by gel filtration on G-25 Sephadex at 4°C. In the eluted protein fractions > 95% of radiolabel could be precipitated using perchloric acid, indicating only slight contamination with unbound nucleotide. Complex concentration varied between 0.112 and 7.7 nM depending upon the tumor line, and was incubated in the presence of folate at 37°C. At appropriate times aliquots of reaction mixture were pipetted into ice-cold quench solution. In all experiments, quenched reactions were allowed to stand on ice for 20 min prior to centrifugation (12,000 ***g***, 4°C, 10 min). The supernatant was then filtered through a glass fiber filter (Gelman GF/A) packed into a disposable 1 ml syringe, and radioactivity determined in 0.9 ml of the filtrate.

Affinity purification of thymidylate synthase

Briefly, enzyme from $HxVRC_5$ xenografts was purified by fractionation of 100,000 ***g*** supernatants using solid ammonium sulfate. The 30–70% precipitate was dialyzed, and thymidylate synthase purified using Affigel Blue chromatography followed by elution from an affinity column of 10-formyl-5,8-dideazafolate-Sepharose (a gift from Dr. J. R. Bertino, Yale University School of Medicine) as described by Rode *et al.* [12]. The enzyme was subsequently dialyzed extensively, to remove dUMP which was a potential contaminant, and concentrated using an Amicon Concentrator with a YM10 membrane. This procedure gave >4000-fold purification. Thymidylate synthesis was stabilized by addition of bovine serum albumin (1%) and stored at 4°C.

RESULTS

The rate at which ternary covalent complex formed in cytosols from each of the three xenografts was examined. At saturating concentrations of CH_2-H_4PteGlu, the concentration of FdUMP at which the maximal rate of FdUMP binding was observed, was approx. 200 nM in each tumor preparation (data not shown). The rate of binding at low enzyme concentration, where CH_2-H_4PteGlu and FdUMP concentrations were in excess, was determined in each tumor cytosol. Data are presented for $HxVRC_5$ in Fig. 1. The rate of association (k_a) was low in each sample being 3.4, 4.8 and 10.2 × 10^6 M^{-1} min^{-1} for $HxELC_2$, $HxVRC_5$ and $HxGC_3$ preparations, respectively (Table 1). Because the rate of binding may be altered by catabolism of FdUMP, this was subsequently examined. At pH 7.4 the rate of catabolism varied considerably between these lines being 73.1, 12.0 and 0.51 nmol/mg protein/hr in cytosols from $HxVRC_5$, $HxGC_3$ and $HxELC_2$, respectively. Addition of 5 mM AMP prevented catabolism for at least 20 min in $HxVRC_5$ cytosols (data not shown). In the presence of 1 mM AMP, the rate of

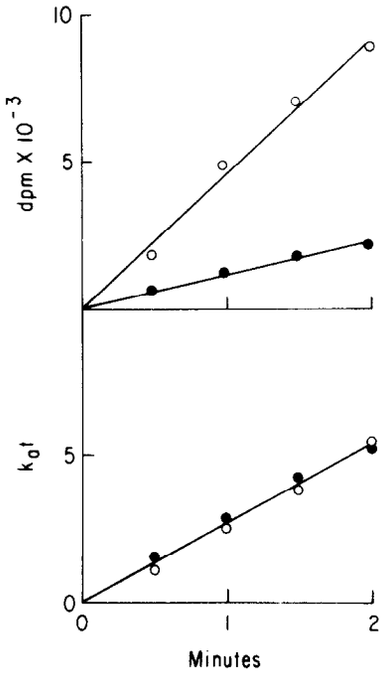

Fig. 1. Top: the rate of [^{3}H]FdUMP binding in $HxVRC_5$ preparations. Final concentrations were 1.99 nM *enzyme binding sites,* 100 μM [6RS]-CH_2-H_4*PteGlu and* (●) 2.86 nM *or* (○) 10.16 nM [^{3}H]*FdUMP. Bottom: replot of the above data according to the second order equation describing the bimolecular rate constant* (k_o) *as described in Materials and Methods, in the presence of added AMP.*

Table 1. Kinetic parameters determined in colon adenocarcinoma xenografts

	Tumor line		
	$HxELC_2$	$HxVRC_5$	$HxGG_3$
K_m dUMP (μ M)	3.6	3.6 ± 0.5[b]	4.2
k_a FdUMP × 10^6 M^{-1} min^{-1}	3.4 ± 0.9	4.8 ± 0.4	10.2 ± 2.6
k_a FdUMP × 10^6 M^{-1} min^{-1} (+ 1 mM AMP)	ND[a]	3.0 ± 0.5	6.9 ± 0.3
K_m [6RS]-CH_2-H_4PteGlu	26.7 ± 2.5	25.0 ± 8	25.0
$t½$ dissociation of complex: no folate (min)	33	33	31

[a]ND = not determined.
[b]S.D. on ≥ 3 determinations.

[^{3}H]FdUMP binding was reduced (Table 1), which may indicate some steric effect of the purine nucleotide. Preliminary data derived using thymidylate synthase purified from $HxVRC_5$ tumors are consistent with the low k_a value (1.01×10^6 M^{-1} min^{-1} determined at 30°C).

We next examined the stability of covalent complex isolated from free ligand by gel filtration using G-25 Sephadex. Complex formed in each cytosol was stable at 0° for the 90-min period examined in the absence of added [6RS]-CH_2-H_4PteGlu. At 37°C the $t½$ for net dissociation was between 31 and 33 min. In all experiments the rate at which complex dissociated was dependent upon the concentration of free CH_2-H_4PteGlu (Fig. 2). The relationship between $t½$ and folate concentration is presented in Fig. 3. Data indicate that complex formed in cytosols from $HxELC_2$ tumors may be stabilized at slightly lower concentrations of (6RS)-CH_2-H_4PteGlu.

The ability of other pteroylmonoglutamates to stabilize covalent ternary complex was also examined. Data for $HxELC_2$ preparations is presented in Fig. 4. Of the folates examined, only H_4PteGlu stabilized the covalent complex significantly. Similar results were obtained with complex derived from $HxVRC_5$ and $HxGC_3$ tumors (data not shown). The data indicate, therefore, that under the experimental conditions used, interconversion of folates to form CH_2-H_4PteGlu occurs at a low rate, if at all.

Other kinetic parameters determined in cytosols are presented in Table 1. The apparent K_m for dUMP was similar in each tumor cytosol (3.6–4.2 μM), and was not altered by addition of 1 mM AMP to the reaction. The apparent K_m for [6RS]-CH_2-H_4PteGlu (25–26.7 μM) was similar in each line, and consistent with data derived using human enzyme [13]. Under the conditions used there appears to be little or no metabolism of CH_2-H_4PteGlu in the presence of FdUMP. After incubation at 37°C for 60 min the concentration of $^{14}CH_2$-H_4PteGlu was similar to that determined before incubation in cytosolic preparations from $HxVRC_5$ tumors (103.2 ± 1.9% of time 0). Preliminary data using thymidylate synthase purified from $HxVRC_5$ tumors by affinity chromatography, gave k_a values of 1.01×10^6 M^{-1} min^{-1} (30°C) and apparent K_m for [6RS]-CH_2-H_4PteGlu of 22.5 ± 3.5 μM, essentially identical to data derived in cytosolic preparations.

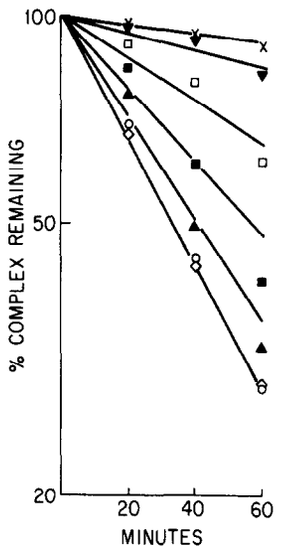

Fig. 2. The stability of FdUMP-thymidylate synthase-CH_2-H_4PteGlu complexes formed in $HxVRC_5$ cytosols with different concentrations of [6RS]-CH_2-H_4*PteGlu. Ternary complexes were isolated by G-25 Sephadex chromatography, and incubated at* 37°C *in the presence of different concentrations of* CH_2-H_4*PteGlu. At the indicated times, aliquots were assayed for residual complex.* (×) 100, (▼) 50; (□) 25; (■) 10; (▲) 5; (◇) 1; (○) 0 μM [6RS]-CH_2-H_4*PteGlu.*

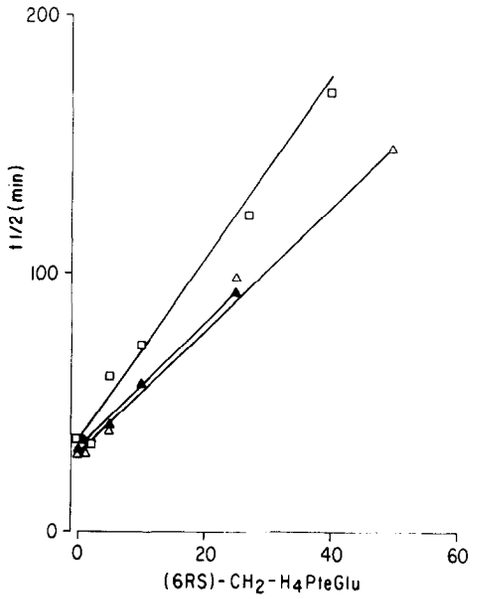

Fig. 3. The relationship between the half-time (t½) *for net dissociation of covalent ternary complex with respect to the concentration of* [6RS]-CH_2-H_4*PteGlu in preparations from* $HxVRC_5$ (▲), *HxELC*$_2$ (□), *and HxGC*$_3$ (△) *tumors.*

DISCUSSION

The purpose of this study was to examine the formation and stability of covalent ternary complex formed between FdUMP, thymidylate synthase and [6R]-CH_2-H_4PteGlu in cytosols derived from tumors that demonstrate different sensitivities to 5-fluoropyrimidines. Although no significant differences between data derived from the sensitive tumor ($HxELC_2$) and intrinsically resistant tumors ($HxGC_3$, $HxVRC_5$) were found, several points are of interest. Of particular importance is the finding that FdUMP binds at a low rate in each tumor cytosol. The highest association rate (k_a) was determined for $HxGC_3$ ($10.2 \pm 2.6 \times 10^6$ M^{-1} min^{-1}), and the lowest k_a was determined in $HxELC_2$ tumors ($3.4 \pm 0.9 \times 10^6$ M^{-1} min^{-1}). In contrast,

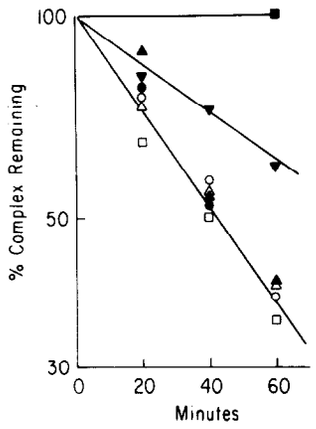

Fig. 4. *Stability of covalent ternary complex in the presence of other folate monoglutamates. Ternary complex was formed in cytosols from HxELC$_2$ tumors, and isolated from free ligands by gel filtration chromatography. Complex was incubated at* 37°C *in the presence of* 50 μM (●) 5-CH$_3$-H$_4$*PteGlu*, (▼) H$_4$*PteGlu*, (○) H$_2$*PteGlu*, (△) 5-CHO-H$_4$*PteGlu and* (▲) *PteGlu, or in the absence of added folate at* 0°C (■) *or* 37°C (□).

k_a values of $2.0 \pm 0.2 \times 10^8$ M^{-1} min^{-1} and $1.4 \pm 0.1 \times 10^7$ M^{-1} min^{-1} have been reported for thymidylate synthase purified from human lymphoblastic leukemia cells (CEM/O) or the same line selected for resistance to 5-fluoro 2′-deoxyuridine (CEM/FdUrd;4). Our data are, however, consistent with values reported for formation of covalent ternary complex with thymidylate synthase purified from human breast cancer cells (k_a, 6.9×10^6 M^{-1} min^{-1}; [14]). Clearly such studies in cytosols may give apparent low rates of association due to rapid catabolism of FdUMP, or metabolism of CH$_2$-H$_4$PteGlu. However, inhibition of FdUMP catabolism did not increase the k_a value in preparations from tumors with catabolic activity. Similarly, the data suggest that under the experimental conditions used (G-25 chromatographed cytosols from HxVRC$_5$ tumors) there was no significant metabolism of CH$_2$-H$_4$PteGlu. Further, in affinity purified enzyme from HxVRC$_5$ tumors that had been dialyzed extensively to remove dUMP, the k_a (at 30°C) was 1.01×10^6 M^{-1} min^{-1}. These data indicate a rate of ternary complex formation in xenografts of colon adenocarcinoma, which is low relative to that reported for enzyme purified from leukemic cells.

In studies described elsewhere [15] we have examined the degree of inhibition of thymidylate synthase and recovery of activity in xenografted tumors after administration of FUra to tumor-bearing mice. In both HxGC$_3$ and HxVRC$_5$ tumors, which are intrinsically resistant to this agent, recovery of thymidylate synthase activity was rapid. We examined, therefore, whether there was a difference in the stability of covalent complex formed in cytosols derived from each of these tumors. In all instances the rate of net dissociation was dependent upon the concentration of free (6RS)-CH$_2$-H$_4$PteGlu, and relatively small differences were determined between tumor complexes. In HxELC$_2$ preparations complex was stabilized to the same extent as complexes from the other tumors, but at slightly lower concentrations of (6RS)-CH$_2$-H$_4$PteGlu. Of the other pteroylmonoglutamates examined, only H$_4$PteGlu stabilized covalent complex. Previously, we have also shown that H$_4$PteGlu stimulated the level of covalent complex formed in the presence of endogenous cofactor [11]. These data suggest that the rate of conversion of PteGlu, H$_2$PteGlu, 5-CHO-H$_4$PteGlu and 5-CH$_3$-H$_4$PteGlu to CH$_2$-H$_4$PteGlu is quite low under the experimental conditions used. Further, the rate of formation of ^{14}C-methionine in HxVRC$_5$ tumors in mice injected with 5-^{14}CH$_3$-H$_4$PteGlu was low [11]. In these experiments the net rate of dissociation was examined, and hence this experiment is not analogous to that of Lockshin and Danenberg [9] in which reassociation of [6-^{3}H]FdUMP was prevented by addition of excess unlabeled ligand. Our experiment was designed to more readily simulate conditions *in vivo* in which the ligand could reassociate with thymidylate synthase. Under these conditions in the absence of added (6RS)-CH$_2$-H$_4$PteGlu, complex from each tumor dissociated at the same rate ($t½$ = 31–33 min). The $t½$ could be increased to 100 min by 20 μM [6RS]-CH$_2$-H$_4$PteGlu in HxELC$_2$ preparations and 40 μM in preparations from HxGC$_3$ and HxVRC$_5$ tumors. It is of note that in the study by Bapat *et al.* [4], a concentration of free [6R]-CH$_2$-H$_4$PteGlu of approx. 300 μM was required to increase the $t½$ for dissociation to 100 min. Our data suggest that net dissociation may be significantly slowed if the concentration of [6R]-CH$_2$-H$_4$PteGlu could be increased to between 10 and 20 μM. Whether this may be achieved *in situ* remains to be determined. However, preliminary studies in which high doses of leucovorin have been administered with FUra, have demonstrated potential in increasing response rates for FUra in the treatment of colorectal adenocarcinoma in man [16–18].

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