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Effect of folate diastereoisomers on the binding of 5-fluoro-2'-deoxyuridine-5'-monophosphate to thymidylate synthase

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Abstract—A series of natural and unnatural stereoisomers of reduced folate coenzymes have been studied for their capacity to facilitate binding of 5-fluoro-2'-dUMP (FdUMP) to bacterial thymidylate synthase (TS). The natural cosubstrate for the enzyme, (6*R*)-5,10-methylene-tetrahydrofolate (CH_2-H_4-folate), was 4-fold more potent than the unnatural 6*S*-form in promoting FdUMP binding to TS, but in a racemic mixture the effect of the 6*R*-form was not affected by the 6*S*-form. FdUMP binding to TS was also stimulated by tetrahydrofolate and dihydrofolate (85% and 30% as compared to (6*RS*)-CH_2-H_4-folate, respectively), but not by the stereoisomers of 5-methyl-tetrahydrofolate and 5-formyl-tetrahydrofolate (leucovorin). These results suggest that folates, which are not a natural cosubstrate for TS, have an additional role in facilitating FdUMP binding to TS.

One of the key enzymes in *de novo* pyrimidine nucleotide synthesis, thymidylate synthase (TS*), requires the reduced folate (6*R*)-5,10-methylene-tetrahydrofolate (CH_2-H_4-folate) as cofactor for the conversion of dUMP into dTMP [1]. The pyrimidine nucleotide analog 5-fluoro-2'-dUMP (FdUMP) is a potent inhibitor of TS, through formation of a stable ternary complex with the enzyme and (6*R*)-CH_2-H_4-folate. This is the basis for anticancer treatment protocols with 5-fluorouracil (5FU) and 6*RS*-5-formyl-tetrahydrofolate [5-HCO-H_4-folate, leucovorin]. 5FU is converted to FdUMP, while (6*RS*)-5-HCO-H_4-folate is metabolized to (6*R*)-CH_2-H_4-folate. The capacity of (6*RS*)-CH_2-H_4-folate to act as a cosubstrate in FdUMP binding to TS has been well established; for other folate intermediates formed during metabolism of (6*RS*)-5-HCO-H_4-folate to (6*R*)-CH_2-H_4-folate this function is less well established. These folate metabolites might also support the formation of a ternary complex of FdUMP with TS [2]. The characteristic of folate-dependent FdUMP binding to TS has been applied in assays by Spears *et al.* [3] and Priest and Doig [4] to measure total cellular and tissue folate pools. Our assays were based on these studies. In contrast to various studies with different folates and bacterial TS, we concentrated our study on (6*RS*)-5-HCO-H_4-folate metabolites, including several stereoisomers, and tested in a cell-free system with purified bacterial TS whether each of the different folates facilitated FdUMP binding to TS.

Materials and Methods

Materials. Lactobacillus casei TS, purified enzyme was obtained from Biopure (Boston, MA, U.S.A.); (6*RS*)-CH_2-H_4-folate and dihydrofolate (H_2-folate) were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). (6*S*)-CH_2-H_4-folate, (6*R*)-CH_2-H_4-folate, (6*S*)-5-methyl-tetrahydrofolate (CH_3-H_4-folate), (6*R*)-CH_3-H_4-folate and (6*R*)-5-HCO-H_4-folate were obtained from Sapec S.A. (Barengo, Lugano, Switzerland). (6*S*)-5-HCO-H_4-folate was kindly provided by Lederle Cyanamid (Etten-Leur, The Netherlands). [6-^{3}H]FdUMP (sp. act. 20 Ci/mmol) was purchased from Moravek Biochemicals Inc. (Brea, CA, U.S.A.). All other chemicals were of analytical grade.

FdUMP ligand binding assay. The various folates were solubilized in buffer A, consisting of 9 mL 0.2 M Tris–HCl (pH 7.2), 1 mL 10 mg/mL bovine serum albumin, 500 μL 1 M ascorbic acid (in 1 N NaOH) and 7 μL 98% mercapto-ethanol. Formaldehyde (26 μL of a 37% solution) was added to a (6*RS*)-5,6,7,8-tetrahydrofolate (H_4-folate) (Sigma) solution in order to prepare the standard (6*RS*)-CH_2-H_4-folate solution [5]. The different folate solutions were stored at −20°, while protected against light. Several dilutions were made from the standard (6*RS*)-CH_2-H_4-folate solution in order to achieve 0.1–50 μM final concentrations in the assay mixture. These diluted solutions were used for the standard curve of folate/FdUMP binding to bacterial TS. The other folates (6*S*)-CH_2-H_4-folate, (6*R*)-CH_2-H_4-folate, H_2-folate, (6*RS*)-H_4-folate, (6*S*)-CH_3-H_4-folate, (6*R*)-CH_3-H_4-folate, (6*RS*)-5-HCO-H_4-folate, (6*R*)-5-HCO-H_4-folate and (6*S*)-5-HCO-H_4-folate were used in several dilutions resulting in final concentrations in the reaction mixture of 0.1–5 μM. The FdUMP ligand binding assay was performed essentially as described previously for the mammalian enzyme [3, 6]. The reaction mixture consisted of 10 μL bacterial TS (25 pmol), 50 μL of one of the folate solutions, 50 μL [6-^{3}H]FdUMP (5 pmol, 0.10 μCi) and 100 μL buffer B (200 mM Tris–HCl, 100 mM NaF, 40 mM mercapto-ethanol, 15 mM CMP and 1% w/v sodium ascorbate, pH 7.4). This mixture was incubated at 30° for 15 min, then 1 mL ice-cold 3% acid-activated charcoal was added in order to remove the free [6-^{3}H]-FdUMP. After mixing the samples were centrifuged for 10 min at 7000 *g*. Radioactivity was measured in 500 μL of the supernatant by liquid scintillation counting. Blanks consisted of the same reaction mixture without addition of folates; they represent the binary complex formation. The results are expressed in fmoles [6-^{3}H]FdUMP binding per mole TS in relation to the concentration of folates.

Results

Maximal FdUMP binding was observed with the racemic mixture of (6*RS*)-CH_2-H_4-folate prepared from (6*RS*)-H_4-folate. This standard curve of (6*RS*)-CH_2-H_4-folate showed a saturation at a concentration higher than 5 μM (not shown). Comparison between the *S*-form and *R*-form of CH_2-H_4-folate demonstrated that the *R*-form had a much higher affinity (4-fold) for the enzyme than the *S*-form (Fig. 1). In addition, (6*RS*)-H_4-folate was a rather good cofactor to support FdUMP binding to bacterial TS with about 85% of the maximal binding measured with (6*RS*)-CH_2-H_4-folate, at equimolar concentrations of the folates (Fig. 2). The use of H_2-folate as cosubstrate resulted in 30% of the maximal binding (Fig. 2). (6*R*)-CH_3-H_4-folate and (6*S*)-CH_3-H_4-folate had a very low affinity for FdUMP-TS while there was no significant difference between the

* Abbreviations: TS, thymidylate synthase, EC 2.1.1.45; CH_2-H_4-folate, 5,10-methylene-tetrahydrofolate; FdUMP, 5-fluoro-2'-dUMP; 5FU, 5-fluorouracil; H_4-folate, 5,6,7,8-tetrahydrofolate; H_2-folate, dihydrofolate; CH_3-H_4-folate, 5-methyl-tetrahydrofolate; 5-HCO-H_4-folate, 5-formyl-tetrahydrofolate.

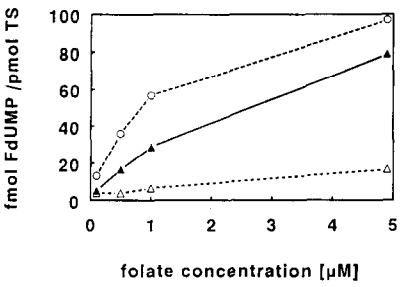

Fig. 1. Comparison of the capacity of (6*R*)-CH_2-H_4-folate (▲), (6*S*)-CH_2-H_4-folate (△) and (6*RS*)-CH_2-H_4-folate (○) to facilitate [^{3}H]FdUMP binding to bacterial TS. For the racemic mixture the total concentration of the *R*- and *S*-form together is shown. Values are from one representative experiment out of five experiments. The standard error of the experimental values was within the range of the symbol.

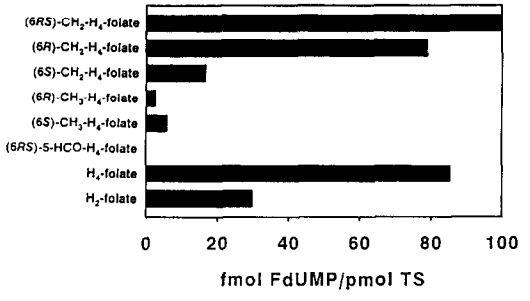

Fig. 2. FdUMP binding to bacterial TS at a final concentration of 5 μM for each of the folates. For the racemic mixtures, the final concentration of the *R*- and *S*-form together was 5 μM. No binding was observed with one of the forms, (6*RS*)-5-HCO-H_4-folate, indicated by the absence of a bar. Values are means of three separate experiments; SD was less than 8%.

isomers (Fig. 2). Neither the *S*-form nor the *R*-form of 5-HCO-H_4-folate facilitated the binding of FdUMP to TS.

Discussion

The preference for (6*RS*)-CH_2-H_4-folate above other folates to facilitate FdUMP binding was consistent with data from Santi *et al.* [2], who used a different assay. Santi *et al.* [2] also tested several folate analogs, developed as an inhibitor of dihydrofolate reductase, but with very low affinity for TS. Studies on the development of antifolates exploiting TS as a target have revealed some of the features which are essential for substrate activity. These include (a) an unsubstituted C^7-N^8 area, (b) the area of attachment of the one carbon unit, the N^5, N^{10}, C^9 atoms, (c) an intact *p*-aminobenzoate moiety and (d) the glutamic acid moiety [7]. Slavik and Zakrzewski [7] demonstrated that substitution of the N^5 atom with a methyl group as in (6*R*)- and (6*S*)-CH_3-H_4-folate or a formyl group as in (6*RS*)-5-HCO-H_4-folate results in loss of substrate activity and for the formyl group in appearance in inhibitory activity. This might explain the low affinity in the FdUMP binding assay of (6*S*)-CH_3-H_4-folate and the very low affinity of (6*RS*)-5-HCO-H_4-folate. H_2-folate and (6*RS*)-H_4-folate have no substitution of the N^5 or N^{10} atom that might decrease their substrate activity [7], but in contrast to (6*RS*)-CH_2-H_4-folate they cannot act as a one carbon donor. H_2-folate lacks a complete aromatic ring; the absence of one H atom at the 5′ and 6′ positions as compared to (6*RS*)-H_4-folate could change the nucleophilicity of N^{10} [7].

Leary *et al.* [8] described the effect of the two stereoisomers of (6*RS*)-CH_2-H_4-folate on the conversion of dUMP into dTMP. The unnatural *S*-form appeared to be a competitive inhibitor of the reaction [8]. We found that the racemic mixture of (6*RS*)-CH_2-H_4-folate facilitated a higher FdUMP binding to TS than (6*R*)-CH_2-H_4-folate at equimolar concentrations, so there is no competitive interaction of the stereoisomers; this can even be considered as a more than additive effect in the FdUMP binding to TS. The (6*RS*)-CH_3-H_4-folate and (6*RS*)-5-HCO-H_4-folate showed such a low affinity for TS, that no significant differences between the isomers could be observed. Balinska *et al.* [9] observed that (6*RS*)-CH_3-H_4-folate was also unable to inhibit the catalytic conversion of dUMP to dTMP.

It is unlikely that the tested folates, except for (6*RS*)-CH_2-H_4-folate, serve as a cosubstrate in the conversion of dUMP to dTMP by TS. However, these compounds may play an inhibitory role in the conversion. This was shown for (6*RS*)-5-HCO-H_4-folate [7] and, especially at high concentrations, (6*R*)-5-HCO-H_4-folate [10]. (6*RS*)-CH_3-H_4-folate did not inhibit TS activity [7]. H_2-folate has a physiological inhibitory function, being a product of the TS reaction. The weak inhibitory properties of H_2-folate (K_i 50 μM for TS) [11] became more important when H_2-folate levels were high, e.g. in cells after exposure to dihydrofolate reductase inhibitors such as methotrexate and trimetrexate [11]. The inhibitory effect increased when H_2-folate was polyglutamated [12]. The carboxyl groups of the glutamate moiety are important for binding of the folate to TS [7].

In conclusion, it has been shown in a well-established test system with purified TS that not only (6*RS*)-CH_2-H_4-folate has affinity for TS but also other folates. These other folates could contribute to the stability of the 5FU-mediated ternary complex formation and also inhibit the conversion of dUMP into dTMP. Both mechanisms add to the modulating capacity of (6*RS*)-5-HCO-H_4-folate on 5FU therapy.

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