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Inhibition of thymidylate synthase by the diastereoisomers of leucovorin

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Summary. The clinical formulation of leucovorin (5-CHO-FH_4) is a mixture of diastereoisomers with markedly different pharmacologic properties. Comparatively little information is available concerning the cellular pharmacology of reduced folate stereoisomers, due largely to the difficulty in preparing sufficient quantities of these compounds for in vitro use. Recent improvements in HPLC technology have now facilitated this process, enabling studies of folate stereochemistry on a larger scale. Using purified (6R) and (6S) leucovorin, we examined the effects of these compounds on the enzymatic activity of *Lactobacillus casei* thymidylate synthase (TS) in a cell-free system. The natural (6S), unnatural (6R), and racemic (6R,S) leucovorin preparations inhibited TS activity by 50% at concentrations of 0.11, 2.1, and 0.52 m*M*, respectively. Dixon plots demonstrated the inhibition to be competitive, with K_i values of 85μ*M*, 1.59 m*M*, and 385μ*M* for (6S), (6R), and (6R,S) leucovorin, respectively. In view of the high doses of leucovorin given clinically and the slow clearance of the unnatural isomer, our observations suggest that leucovorin may have important direct inhibitory effects on folate-requiring enzymes.

Introduction

Leucovorin [(6R,S)-5-formyltetrahydrofolate, LV] is a reduced folate that has been used for many years to prevent host toxicity after intermediate- and high-dose methotrexate therapy. Recent studies have demonstrated that this reduced folate also plays an important role in modulating the cytotoxicity of the pyrimidine antimetabolite 5-fluorouracil (5-FU). Intracellularly, 5-FU is converted to 5-fluorodeoxyuridine monophosphate (5-FdUMP), a potent inhibitor of thymidylate synthase (TS). TS is the only de novo source of thymidylate in the cell and, as such, is a key enzyme in mammalian DNA synthesis. The enzyme catalyzes the reductive methylation of deoxyuridylate by 5,10-methylene tetrahydrofolate to form thymidylate and dihydrofolate. In the presence of sufficient 5,10-methylene tetrahydrofolate, 5-FdUMP forms a covalent ternary complex with TS, binding with a K_d of $1-5 \times 10^{-11}$*M* [13, 17, 18, 21]. In both experimental systems [11, 14, 24, 29, 34] and clinical trials [9, 10, 22, 23, 28], leucovorin has been found to enhance significantly the antitumor activity of 5-FU, due primarily to the expansion of intracellular reduced folate pools, leading to stabilization of the 5-FdUMP-TS-folate ternary complex. The resultant depletion of cellular thymidine pools leads to inhibition of DNA synthesis and cell death.

The clinical formulation of leucovorin is a mixture of stereoisomers around the C6 carbon of the pteridine ring. Most of the biological activity of the compound is presumed to reside in the (6S) isomer. The (6S) and (6R) isomers differ substantially in their cellular pharmacology and clinical pharmacokinetics. At the cellular level, the unnatural (6R) stereoisomer of leucovorin has been shown to be 20 times less effective than the (6S) isomer as a competitive inhibitor of methotrexate transport and 100 times less effective than the (6S) isomer in preventing methotrexate cytotoxicity [25]. It is important to note, however, that the (6R) isomer is not biologically inert and, when present in sufficient concentration, does appear to be able to enter cells and effectively overcome methotrexate toxicity. Recent data suggest that the (6R) isomer of leucovorin acts as a competitive inhibitor of membrane transport of the (6S) isomer and of 5-methyltetrahydrofolate [4].

Following intravenous injection of (6R,S) leucovorin, the (6S) isomer disappears from plasma, with a $t^1/_2$ of approximately 32 min, whereas clearance of the (6R) iso-

Abbreviations: FH_4 – L-tetrahydrofolic acid; FH_2 – 7,8-dihydrofolic acid; CH_2FH_4 – 5,10-methylenetetrahydrofolic acid; 5-CHO-FH_4 – leucovorin, 5-formyl-tetrahydrofolic acid, LV; 10-CHO-FH_4 – 10-formyltetrahydrofolic acid; 5-CH_3FH_4 – 5-methyltetrahydrofolic acid; 5-FdUMP – 5-fluorodeoxyuridine monophosphate; dTMP – deoxythymidine monophosphate; TS – thymidylate synthase; ΔA – change in absorbance; dUMP – deoxyuridine monophosphate; 5-FU – 5-fluorouracil

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Table 1. Effect of leucovorin on thymidylate synthase activity

Leucovorin preparation	Concentration required for 50% enzyme inhibition (m*M*)[a]	K_i (m*M*)
(6S) isomer	0.111	0.085
(6R) isomer	2.14	1.59
(6R,S) leucovorin	0.519	0.385

[a] At 37° C in 0.09 m*M* (6S) CH_2FH_4 and 0.9 m*M* dUMP, the concentration required for 50% inhibition was calculated as described by Cheng and Prusoff [6]

mer is considerably slower, proceeding with a $t^1/_2$ of approximately 7.5 h [27]. Thus, within 2 h of injection of a 50/50 mixture of (6R,S) leucovorin, the (6R) leucovorin concentration in plasma is approximately 100-fold that of (6S) leucovorin, and repetitive dosing quickly leads to accumulation of large quantities of (6R) relative to (6S) leucovorin. The impact of high concentrations of (6R) leucovorin on folate-requiring biochemical pathways has not yet been clearly elucidated. In this study, we report the effects of leucovorin stereoisomers on thymidylate synthase activity in a cell-free system.

Materials and methods

Chemicals. (6R,S) FH_4, (6R,S) 5-CHO-FH_4, folic acid, dUMP, and formaldehyde were obtained from Sigma Chemical Co. (St. Louis, Mo.), and CNBr-activated Sepharose 4B and Sephadex G100 were purchased from Pharmacia (Piscataway, N.J.). Biorad protein reagent was purchased from Biorad (Rockville Center, N.Y.), and other reagents were obtained at the highest available purity from various commercial sources.

Protein determination. During the purification of thymidylate synthase, UV absorbance at 260 and 280 nm was used for the estimation of protein concentrations [32]. The protein concentration of the purified enzyme was determined by the Biorad method at 595 nm.

Purification of thymidylate synthase. The crude extract of thymidylate synthase, prepared from a methotrexate-resistant mutant of *Lactobacillus casei*, was purchased from Biopure (Boston, Mass.). It was purified according to the method of Banerjee et al. [3] using Sephadex G100 and 10-CHO-FH_4 affinity columns. The enzyme was absorbed on the affinity column with 50 m*M* TRIS-HCl (pH 7.2) buffer containing 2 m*M* dithiothreitol, 10% glycerol, and 50 μ*M* dUMP. The purified enzyme was then eluted from the affinity column after the salt concentration had been raised to 0.1 *M* KCl in 0.1 *M* TRIS-HCl (pH 7.5) containing 2 m*M* dithiothreitol, 10% glycerol, and 50 μ*M* dUMP. The purification was 100-fold and the purified enzyme had a specific activity of 2.55 μmol dTMP formed min^{-1}/mg $protein^{-1}$ at 37° C. Bovine serum albumin (1 mg/ml) was added to the purified enzyme to enhance stabilization. The enzyme was stored in 200-μl aliquots at –20° C.

Assay of thymidylate synthase activity. The spectrophotometric assays were performed as described by Wahba and Friedkin [31], with some modifications. The assay buffer consisted of 20 m*M* $MgCl_2$, 1 m*M* EDTA, and 10 m*M* mercaptoethanol in 50 m*M* TRIS-HCl (pH 7.4). After deoxygenation by passage of N_2, it was aliquoted in 4 ml volume and stored at –20° C. A stock solution of (6R,S)CH_2FH_4 was made by mixing together 36 mg (6R,S)FH_4 and 30 μl CH_2O in 14.4 ml assay buffer. After all of the FH_4 had dissolved, 3.6 ml 1 *M* mercaptoethanol was added. The concentration of CH_2FH_4 was calculated from the absorbance at 296 nm using a molar extinction coefficient of 3.2×10^4 [5]. The amount of (6S)CH_2FH_4 was assumed to be half of the racemic mixture. The solution was aliquoted in 1 ml volume and was stable at –20° C for at least 2 months.

For each enzyme assay, the buffer, CH_2FH_4, and dUMP solutions were mixed at 4° C and 120 μl was added to a masked microcuvette that had been deaerated with N_2. The cuvette was sealed with parafilm and preincubated for 10 min at 37° C. The blank cuvette had no enzyme, whereas the sample cuvette received 5–10 μl purified TS (4.05 μg) at time zero to start the reaction at 37° C. The change of absorbance at 340 nm in the sample cuvette was used to monitor the formation of FH_2 from CH_2FH_4 as dTMP was formed from dUMP. The recorder was set at an absorbance range of 0.1 and the absorbance was recorded for 1.5–2 min on a Cary 219 spectrophotometer. The initial rate of reaction was recorded as the change in absorbance at 340 nm min^{-1} ($\Delta A_{340\ nm}$). The molar extinction coefficient for the formation of FH_2 is 6,152 at 340 nm [19].

For the kinetic studies characterizing TS with respect to its substrates, the concentration of dUMP varied from 2.09 to 18.5 μ*M* and that of CH_2FH_4 was 152 μ*M* for determination of Michaelis constants for dUMP. For the determination of Michaelis constants for CH_2FH_4, the concentration of dUMP was 0.9 m*M* and that of CH_2FH_4 varied from 15.5 to 152 μ*M*. To determine the effect of leucovorin on the catalytic activity of TS, varying concentrations of leucovorin were incubated in a masked microcuvette at 37° C for 10 min with the assay buffer, 0.9 m*M* dUMP, and CH_2FH_4, at concentrations ranging from 31 to 152 μ*M*. The total volume of the mixture was 120 μl. TS was added at time zero and the rate of the reaction was followed spectrophotometrically as described above. The Michaelis constants K_m and V_{max} were derived from the raw data by using the Enzfitter program developed by R. J. Leatherbarrow (Elsevier, Amsterdam, The Netherlands). The values of the constants were reported as means ± standard error.

Preparation of (6R) and (6S) 5CHO-FH_4

Racemic 5CHO-FH_4 at 1 mg/ml was separated into its (6R) and (6S) stereoisomers by HPLC using a bovine serum albumin-bonded silica column as previously described [7]. Baseline separation of the isomers is achieved with this process, and fractions were collected only when there was no potential for overlap of the stereoisomer peaks. The pooled samples of pure isomers were lyophilized and dissolved in a minimal amount of water at room temperature. The resultant solution was cooled in ice, and the large amount of sodium phosphate precipitate from the HPLC mobile phase was then removed by centrifugation. The residual amount of sodium phosphate in the supernatant was removed by elution through a Supelclean C-18 solid-phase extraction cartridge obtained from Supelco (Bellafonte, Pa). The cartridge was conditioned sequentially with 4 ml methanol, 4 ml water, and 2 ml of 0.37 *M* sodium phosphate at pH 7. Approximately 6 mg 5CHO-FH_4 was then eluted with 6 ml methanol. The methanol was eliminated by evaporation under nitrogen and the residual 5CHO-FH_4 was dissolved in assay buffer. Its concentration was determined by absorbance at 285 nm using a molar extinction coefficient of 3.72×10^4 [30]. Injection of aliquots of each pure stereoisomer preparation on the chiral HPLC system yielded only a single peak at the appropriate retention time. The folate solution was stored at –20° C.

Results

The steady-state kinetic calculations for the reaction of thymidylate synthase with dUMP and CH_2FH_4 in a pH 7.4 buffer at 37° C gave apparent Michaelis constants (K_m) of 1.98 ± 0.13 and 47.6 ± 8.2 μ*M* for dUMP and CH_2FH_4, respectively. The V_{max} values were 5.6 ± 0.1 and 9.0 ± 0.69 μ*M* min^{-1} for the two substrates, respectively.

The inhibition of TS by leucovorin varied depending on the stereo configuration of the isomer. In 0.9 m*M* dUMP and 0.33 m*M* (6R,S)CH_2FH_4, 0.64 m*M* (6S) 5-

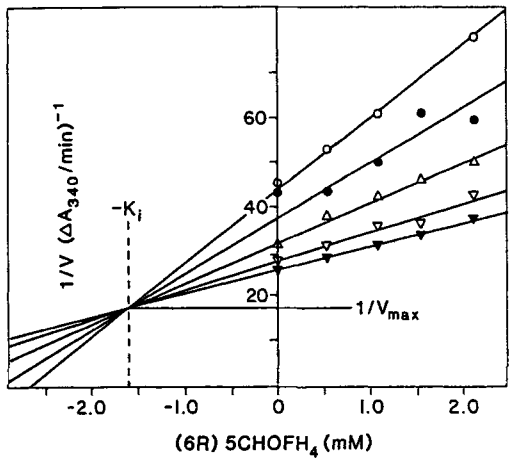

Fig. 1. Dixon plot for (6R) CHO-FH_4 inhibition of TS. The enzyme and dUMP concentrations were 4.6 × 10^{-7} *M* and 0.9 m*M*, respectively. The inhibitor concentration varied from 0 to 2.11 m*M*. The μ*M* concentrations of CH_2FH_4 were (▼) 77.5, (▽) 62, (△) 49.6, (●) 37.2, and (○) 31

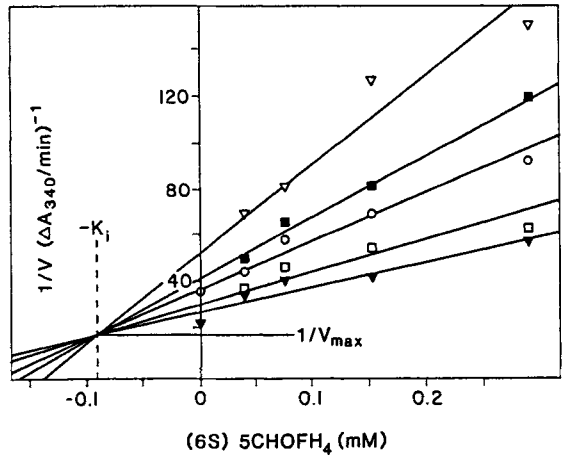

Fig. 2. Dixon plot for (6S) CHO-FH_4 inhibition of TS. The enzyme and dUMP concentrations were 4.6 × 10^{-7} and 0.9 m*M*, respectively. The inhibitor concentration varied from 0 to 290 *M*. The μ*M* concentrations of CH_2FH_4 were (▼) 152, (□) 77.5, (○) 49,6, (■) 37.2, and (▽) 31

CHO-FH_4 inhibited the reaction by 60% but equimolar (6R) isomer had no effect. The concentration of leucovorin diastereoisomers required for 50% enzyme inhibition are shown in Table 1 [6]. The concentration of (6S) isomer required for 50% inhibition was approximately 20 times less than that of the (6R) isomer. Although the (6R) isomer was the less effective inhibitor, it appeared to interfere with the interaction of the (6S) isomer with TS since the racemic mixture of leucovorin was about 5 times less effective as an inhibitor than was the (6S) isomer.

The inhibition of thymidylate synthase activity by 5CHO-FH_4 was found to be competitive with respect to CH_2FH_4. The Dixon plots gave K_i values of 1.59 m*M*, 85 μ*M* and 385 μ*M* for (6R), (6S), and (6R,S) 5CHO-FH_4, respectively. Figures 1 and 2 show the Dixon plots for (6R) and (6S) 5CHO-FH_4 inhibition.

Discussion

The observation that leucovorin can effectively enhance the cytotoxicity of 5-FU in both the laboratory and the clinic has led to a resurgence of interest in the pharmacology of reduced folates. The clinical formulation of leucovorin is a mixture of diastereoisomers with markedly different pharmacologic properties. Indeed, administration of this drug is equivalent to treatment with two chemically related compounds that have the potential to undergo multiple interactions. At the present time, comparatively little information is available concerning the cellular pharmacology of reduced folate stereoisomers, due largely to the difficulty in preparing sufficient quantities of these compounds for in vitro use. Recent improvements in HPLC technology have now facilitated this process, enabling studies of folate stereochemistry on a larger scale.

Available information suggests that the biological activity of reduced folate stereoisomers varies considerably, depending on the specific folate as well as the enzymatic reaction or transport process involved. Whereas the unnatural isomer of leucovorin is less effectively transported into cells than the natural (6S) isomer [4, 25], the stereoisomers of 5-methyl tetrahydrofolate influx at nearly equivalent K_m values of 2.5 and 3.5 μ*M*, respectively, for the natural and unnatural forms [33]. Of particular interest is the observation that the unnatural isomer of tetrahydrofolate, and perhaps other reduced folates, is a substrate for mammalian folylpolyglutamate synthetase [20]. The intracellular accumulation of folate polyglutamates in the unnatural configuration could have profound influences on folate metabolic pathways. Unnatural folate isomers can also behave as inhibitors of enzymes for which the natural isomer is a substrate. Such appears to be the case for methylene tetrahydrofolate in its interaction with thymidylate synthase [15].

In view of the complex interconversions of folates that occur intracellularly, we decided to examine the effects of leucovorin stereoisomers on TS in a cell-free system. The enzyme used in this study had apparent K_m values of 1.98 and 47.6 μ*M* for dUMP and CH_2FH_4, respectively. These values are similar to those previously reported for *L. casei* TS by other investigators [8].

Slavik and Zakrzewski [26] have previously demonstrated that (6R,S) 5-CHO-FH_4 inhibited TS from *E. coli* noncompetitively with respect to CH_2FH_4, with a K_i of 250 μ*M* at 20° C. More recently, Friedkin and colleagues [12] found that (6S) 5-CHO-FH_4 inhibited *E. coli* TS competitively versus CH_2FH_4 at room temperature or 30° C. The latter authors reported that 0.2 m*M* (6S) 5-CHO-FH_4 gave 50% inhibition of TS at CH_2FH_4 and dUMP concentrations of 50 and 16.7 μ*M*, respectively. No previous studies have examined the effects of (6R) 5-CHO-FH_4 on the enzymatic activity of TS. In the present study, we have observed 50% inhibition of TS at 0.52, 0.11, and 2.1 m*M* concentrations of (6R,S), (6S), and (6R) 5-CHO-FH_4, respectively. Inhibition by the pure isomers and racemate were competitive with respect to CH_2FH_4. Of interest is the observation that although the (6R) isomer is considerably less potent than the (6S) isomer as an enzyme inhibi-

tor, it does appear to interfere with the interactions of the (6S) isomer with TS, since the racemic mixture of leucovorin is approximately 5 times less effective as an enzmye inhibitor than is the (6S) isomer.

The precise mechanism by which leucovorin interferes with TS activity is unknown, although it likely behaves as a quasi-substrate to bind TS in a ternary complex with dUMP [16]. The potency of leucovorin as an inhibitor of TS could potentially be significantly enhanced intracellularly by conversion to polyglutamate derivatives. Indeed, it is well known that polyglutamates of methotrexate [2] and of CH_2FH_4 [24] bind to TS with much greater affinity (as much as 1,000-fold) than do the corresponding monoglutamate forms.

Our observations that the stereoisomers of leucovorin interact directly with TS are of particular importance in view of the high doses of leucovorin now given in clinical practice. Peak plasma concentrations of 100 μM (6R,S) leucovorin are easily obtained, and high concentration ratios of (6R):(6S) leucovorin exist following intravenous administration [27].

In view of the fact that leucovorin isomers interact directly with TS, it is also important that the effects of these compounds on FdUMP binding to TS be examined. The accumulation of high concentrations of (6R) leucovorin in plasma following administration of high drug doses could potentially interfere with the formation or stability of the FdUMP-TS-folate ternary complex and impair the effectiveness of 5-FU chemotherapy.

Leucovorin can rescue cells from the toxic effects of methotrexate in a competitive manner [1]. Since leucovorin provides a source of reduced folates to bypass the block in dihydrofolate reductase activity, the competitive nature of rescue cannot be easily explained by the repletion of reduced folate pools. However, it could be explained by competition between methotrexate and leucovorin for binding at key enzymatic sites such as TS, dihydrofolate reductase, glycinamide ribotide transformylase, aminoimidazole carboxamide ribotide transformylase, or folylpolyglutamate synthetase. The effects of leucovorin and its isomers on the latter enzymes require further study.

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