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ANTITUMOUR EFFECTS OF PURE DIASTEREOISOMERS OF 5-FORMYLTETRAHYDROFOLATE IN HEPATIC TRANSPLANTS OF A RODENT COLON CARCINOMA MODEL

GÖRAN CARLSSON,* BENGT GUSTAVSSON,* ROLAND FRÖSING,* ELISABETH ODIN,* LARS-OLOF HAFSTRÖM,† C. PAUL SPEARS‡ and PER-ANDERS LARSSON§

*Department of Surgery, Östra Sjukhuset, Göteborg, Sweden; †Department of Surgery, Norrlands University Hospital, Umeå, Sweden; and ‡Comprehensive Cancer Center, University of Southern California, Los Angeles, CA U.S.A.

Abstract—The effects of the two diastereoisomers of 5-formyltetrahydrofolate on tumour growth, thymidylate synthase (TS, EC 2.1.1.45) levels, and potentiation of 5-fluorouracil cytotoxicity were studied in an *in vivo* rat colon carcinoma model, transplanted to liver. The animals were randomized into eight groups, treated with daily i.v. tail vein injections of racemic (d,l)-5-formyltetrahydrofolate (5-CHO-FH_4), 15 mg/kg, (l)-5-CHO-FH_4 7.5 mg/kg, and (d)-5-CHO-FH_4 7.5 mg/kg, 5-fluorouracil (FUra) 30 mg/kg, (d,l)-5-CHO-FH_4 15 mg/kg + FUra 30 mg/kg, (l) 5-CHO-FH_4 7.5 mg/kg + FUra 30 mg/kg, and (d)-5-CHO-FH_4 7.5 mg/kg + FUra 30 mg/kg, and a sham-treated control group. The average tumour size of the groups was equal at the start of treatment. After six days' treatment the average tumour sizes were at laparotomy 3.3 ± 1.0 g in the (d/l)-5-CHO-FH_4 treated group, compared to 2.0 ± 0.1 g in the FUra treated group and 7.1 ± 3.1 g in the controls. Natural (l)-5-CHO-FH_4 promoted tumour growth (average tumour weight 10.8 ± 4.0 g), whereas the unnatural (d)-5-CHO-FH_4 alone retarded it (average tumour weight 1.2 ± 0.40 g). (l)-5-CHO-FH_4 induced a significant increase in tumour tissue TS levels by [^{3}H]FdUMP radioligand assay (27.5 ± 8.4 pmol/g tumour tissue) compared to controls (16.8 ± 6.1 pmol/g tumour tissue). Increases in 5,10-methylenetetrahydrofolate and tetrahydrofolate occurred with FUra alone, with a further statistically significant increase in both folates with the addition of (d)-5-CHO-FH_4 to FUra.

Key words: 5-formyltetrahydrofolate; 5-fluorouracil; tumour growth; diastereoisomers; thymidylate synthase

Biomodulation of FUra with folates has in recent years been seen as holding promise for the improvement of the antitumour activity of FUra[‖] [1–6]. (d,l)-5-formyltetrahydrofolate (5-CHO-FH_4, folinic acid) is widely used in anticancer therapy as a folate modulator for fluoropyrimidines. The rationale is an increased inhibition of the key *de novo* enzyme thymidylate synthase (TS, EC 2.1.1.45) [7]. The natural substrates for TS are 2′-deoxyuridine 5′-monophosphate (dUMP) and the one-carbon donor 5,10-methylenetetrahydrofolate (CH_2FH_4). The combination of the active nucleotide of FUra, FdUMP, with TS and CH_2FH_4 forms an inhibitory covalent ternary complex that requires CH_2FH_4 in large stoichiometric excess to ensure maximal TS inhibition [8]. A lack of reduced folates is potentially one of the most important factors in explaining the poor efficiency of TS inhibition by FdUMP and resistance to FUra-based chemotherapy [9, 10]. Folate deficiency can be circumvented by the addition of 5-CHO-FH_4 to FUra. 5-formyltetrahydrofolatecalcium (d/l)-5-CHO-FH_4 is an equimolar mixture of the two pteridine ring C_6 diastereoisomers.

It has been stated that only the l-form of 5-formyltetrahydrofolate is biologically active and converted into active folate cofactors (i.e. 6(l)-CH_2FH_4 [11, 12]). It has recently been reported, however, that the d-isomers are not inert and that the unnatural folates can compete with the l-isomers for intracellular transport and interfere with their intracellular metabolism, acting at various enzymatic levels [13–15].

We have developed a rat colon carcinoma model that has been useful for exploring the *in vivo* pharmacodynamics of FUra/folate therapy [16]. These tumours grow as a well-defined solid tumour mass in the liver, and are repeatedly measurable. Assessment of tumour masses at different stages is used to evaluate the effectiveness of different therapeutic modalities, and the tumours are modestly sensitive to FUra. Growth and pharmacodynamic behaviour of these experimental tumours has proven stable during repeated passages and assays (over 85 passages) [16, 17].

The aim of the present study was to evaluate the effects of the racemic (d/l)-5-CHO-FH_4 and the individual d- and l-forms of 5-CHO-FH_4 in combination with FUra on experimental tumour growth and levels of TS.

MATERIALS AND METHODS

Animals and experimental model

Female Wistar rats were anaesthetised with diethyl-ether inhalation, and the liver exposed via a midline laparotomy. 1.0×10^6 viable cells, trypsinized and immersed in DMEM, of a transplantable N-methyl-N′-nitrosoguanidine-induced tumour (NGW) were inoculated in the subcapsular space of the right liver lobe. Inoculation of precise numbers of cells was done by trypsinization followed by negrosin staining and viability counting in a Bürker chamber of freshly passaged cells. Tumour

§ Corresponding author: Per-Anders Larsson, M.D., Ph.D., Department of Surgery, Östra sjukhuset, S-416 85 Göteborg, Sweden. Tel. 46 31 37 40 00; FAX 46 31 25 14 63.

‖ Abbreviations: NGW, nitrosoguanidine induced adenocarcinoma; FH_4, H_4-pteroylglutamate$_n$; CH_2FH_4, 5,10-CH_2H_4-pteroylglutamate$_n$, 5-CHO-FH_4, 5-formyl-H_4-pteroylglutamate$_n$ (i.e. leucovorin); FUra, 5-fluorouracil; TS, thymidylate synthase; and SHMT, serine hydroxymethyltransferase.

take was 100%. The NGW tumour is a rapidly growing poorly differentiated colon carcinoma with a volume doubling time of approximately 24 hours. Relaparotomy was performed at day eight and the longest (A) and the perpendicular (smallest) (B) diameters of the liver tumours were calculated according to the approximation formula,

$$V = A \times B^2 \times 0.5.$$

Treatment was given by tail vein as bolus injections over 2 minutes, daily, days 9 to 11 and 14 to 16. Treatment was always given between 9:00 and 11:00 a.m. to avoid effects of diurnal variation [18]. 5-CHO-FH_4 preparations were given in the same vein as a bolus injection within 30 seconds after FUra.

The animals were killed at day 17. The body weight and tumour volume were registered, the tumour mass excised, and the tumour wet weight registered. Tumour biopsies were stored at −70°C until the analyses of TS and reduced folates were performed.

Experimental design

One hundred twenty rats were inoculated with the NGW tumour in the liver, and then randomized at day 8 to the eight different treatment groups. One group (n = 15 animals) received (d,l)-5-CHO-FH_4 15 mg/kg; one group (n = 25 animals) received FUra 30 mg/kg; one group (n = 15 animals) FUra 30 mg/kg, and (d,l)-5-CHO-FH_4 15 mg/kg; one group (n = 10 animals) received (l)-5-CHO-FH_4, 7.5 mg/kg; one group (n = 10 animals) received (d)-5-CHO-FH_4 7.5 mg/kg; one group (n = 10 animals) received FUra 30 mg/kg, and (l)-5-CHO-FH_4 7.5 mg/kg; one group (n = 10 animals) received FUra 30 mg/kg, and (d)-5-CHO-FH_4 7.5 mg/kg; and the control group (n = 25 animals) received sham injections of saline 0.1 mL. There were no significant differences in the day 8 tumour volume or body weight among the eight experimental groups when the treatment started (Table 1).

Chemicals

FUra, (d,l)-5-CHO-FH_4, (l)-5-CHO-FH_4, and (d)-5-CHO-FH_4 were supplied by Lederle Cyanamid, Wayne, NJ. Pure diastereoisomers of 5-CHO-FH_4 were 99% free of the other diastereoisomer. The doses of FUra and 5-CHO-FH_4 were chosen according to initially performed animal toxicity testing to achieve maximal antitumour effect without lethality to the rats.

L. Casei TS as an ammonium sulfate preparation (Biopure, Boston, MA) was stored at −20°C with an equal amount of glycerol. Serial dilution of enzyme for the TS and folate assays was done in homogenization buffer (see below), supplemented with 0.2% BSA. The initial concentration was 1200 mIU/mL.

Table 1. Animal weights and tumour volumes at start of drug therapy at day 8 (means ± SD; n = number of rats)

Treatment group	n	Animal weight (g)	Tumour volume (mm^3)
Control	25	199 ± 2.6	261 ± 77.0
FUra	25	198 ± 2.5	260 ± 151.0
d,l-5-CHO-FH_4	15	200 ± 2.3	261 ± 77.2
FUra + d,l-5-CHO-FH_4	15	199 ± 1.7	259 ± 86.2
l-5-CHO-FH_4	10	200 ± 4.4	261 ± 47.9
FUra + l-5-CHO-FH_4	10	198 ± 4.2	250 ± 72.4
d-5-CHO-FH_4	10	198 ± 3.2	257 ± 57.5
FUra + d-5-CHO-FH_4	10	198 ± 3.4	261 ± 30.5

[6-^{3}H]FdUMP (specific activity of 18–20 Ci/mmole) was purchased from Moravek Biochemicals®, Brea, CA. Purification of radiolysis breakdown products was carried out monthly by DEAE-column separation [16].

Homogenization buffer (100 mL) was prepared by adding 550 mg 5′-CMP, 420 mg sodium fluoride, 28 μL 2-mercaptoethanol, 1 g Na-ascorbate (pH 7.0), and 200 μg BSA to 0.18 M Tris-HCl buffer, pH 7.4. *Folate buffer* was prepared by adding 52.8 μL of 14.3 M 2-mercaptoethanol, 26.4 μL of 37% (w/w) formaldehyde, and 22.752 mL of homogenization buffer, pH 7.4, to 25 mg (d,l)-FH_4 (Sigma, St. Louis, MO). The mixture was incubated for 10 min at room temperature (in the dark), which allowed completion of CH_2FH_4 formation. All chemicals used for preparation of these buffers were purchased at the highest available grade from different commercial sources.

Analysis of thymidylate synthase and reduced folates

The TS binding activity and concentration of CH_2FH_4 and FH_4 in the NGW tumour was measured at day 17, 24 hours after the last drug injection.

Portions of frozen tumour tissue were weighed and minced with scissors in a 10-fold excess of homogenization buffer at 4°C. Homogenates were prepared by the action of rotating shearing blades (Turrax) for 30 sec at 4°C.

The [^{3}H]FdUMP ligand-binding assay was used for analysis of free TS [19]. The objective was to compare intratumoural free TS levels in the drug administered to the control animals, and subsequently analysis of total TS in the tumour specimens was not performed. One mL of the homogenate (duplicate samples) was centrifuged at 4°C and 4000 × g for 20 min. To 50 μL of the cytosol was added 25 μL of homogenization buffer, 50 μL [^{3}H]FdUMP (approximately 200.000 dpm) in H_2O, and 25 μL of 46.2 μmol CH_2FH_4. These procedures were carried out at 2–4°C. The tubes were incubated at 37°C for 10 min, then 3 mL of 3% acid charcoal (rapidly stirred at 4°C) was added. This was followed by vortex mixing and centrifugation at 4°C and 4000 × g for 15 minutes to precipitate unbound radioactivity; then 0.8 mL of the supernatant was subjected to beta scintillation counting. TS concentrations are expressed as pmole bound FdUMP per gram tissue (wet weight).

Tumour tissue levels of CH_2FH_4 and FH_4 were assayed by adopting the methodology of Priest with some modifications [8, 19–21]. To 50 μL aliquots of cytosol or homogenization buffer alone (blank) were added 50 μL of the [^{3}H]FdUMP (approximately 20 pmoles or 800.000 dpm), 4 pmoles of *L. Casei* TS (×1.7 = 6.8 pmoles of FdUMP binding sites) in 25 μL of homogenization buffer (with 0.2% BSA), and 25 μL of folate buffer (above), with or without formaldehyde for estimation of FH_4 + CH_2FH_4 and 5,10-CH_2H_4, respectively. These procedures were carried out at 2–4°C. The tubes were then incubated at 37°C for 10 min, and 1 mL of ice-cold 3% acid charcoal was added to separate protein-bound radioactivity. The samples were centrifuged at 4°C and 4000 × g for 15 minutes, and scintillation counting performed. Folate concentrations are expressed as nanomoles per gram tissue (wet weight).

Statistics

Standard parametric test (i.e. Student's *t*-test), one-way ANOVA with the Scheffe *F*-test, and regression analysis were performed to describe and compare data using the StatView software package. Data are presented as arithmetic means ± standard deviations (SD). $P \leq 0.05$ was considered statistically significant.

RESULTS

Antitumour effects

Tumour wet weights are presented in Fig. 1. Tumour volumes (Table 2) at day 17 correlated with tumour weight ($r = 0.896$, Fig. 1). The tumours in rats treated with FUra were significantly smaller than in the controls, 2.03 ± 0.98 g compared to 7.09 ± 3.08 g. When FUra and (d,l)-5-CHO-FH_4 were given together, the inhibition of tumour growth was more pronounced, but not statistically significant compared to FUra alone. The tumours in the animals treated with (d,l)-5-CHO-FH_4 only were significantly smaller than the control tumours (3.29 ± 0.959 g), although the growth inhibition induced by 5-formyltetrahydrofolate alone was not as pronounced as the FUra effect.

l-5-CHO-FH_4 had a statistically significant growth-stimulating effect (10.81 ± 4.03 g). In contrast, single agent d-5-CHO-FH_4 had a statistically significant growth-inhibiting effect *per se*, with a mean tumour weight of 0.60 ± 0.20 g. Addition of l-5-CHO-FH_4 to FUra increased growth inhibition, but this did not reach statistical significance compared to FUra alone, nor did the addition of d-5-CHO-FH_4 to FUra.

Drug toxicity

No mortality was associated with any of the treatments. Rats treated with FUra therapy showed epiphoria (tearing or excess of lacrimation), whereas the untreated control animals showed no sign of health deterioration, despite increasing tumour masses. Adverse clinical signs could not be observed in the animals treated with (d,l)-5-CHO-FH_4 or (l)-5-CHO-FH_4 only, despite the lack of weight gain in the (l)-5-CHO-FH_4-treated group (Tables 1 and 2). (d)-5-CHO-FH_4 was mildly toxic as judged by the animal weight at day 17, which was significantly lower than the control animal weight.

Table 2. Response and toxicity of the treatment, animal weight, and tumour volume at termination of drug therapy at day 17 (means ± SD). *n* = number of surviving rats at the end of the experiment. Therapy was given days 9–11 and 14–16 by means of rapid bolus i.v. tail injections. All comparisons were made to control rats, and * indicates statistical significance ($P \leq 0.05$).

Treatment group	*n*	Animal weight (g)	Tumour volume (mm^3)
Control	25	209 ± 4.1	6307 ± 1571
FUra	25	179 ± 11.3*	2184 ± 1591*
d,l-5-CHO-FH_4	15	208 ± 3.0	5916 ± 2485
FUra + d,l-5-CHO-FH_4	15	187 ± 2.5*	1678 ± 1100*
l-5-CHO-FH_4	10	198 ± 9.6	7691 ± 4266
FUra + l-5-CHO-FH_4	10	173 ± 11.5*	1291 ± 783*
d-5-CHO-FH_4	10	187 ± 2.9*	1027 ± 428*
FUra + d-5-CHO-FH_4	10	170 ± 8.9*	475 ± 157*

TS levels

In the control animals, TS levels averaged 16.8 ± 6.14 pmole/g. In the animals treated with (l)-5-CHO-FH_4, the TS levels were significantly increased (27.5 ± 8.36 pmole/g; see Table 3). For the other treatments, the differences in TS levels compared to controls were not statistically significant, although the free TS levels were decreased after treatment with FUra. The combination of FUra and (d)-5-CHO-FH_4 increased intracellular CH_2FH_4 concentrations significantly compared to the controls, 5.64 ± 5.50 vs 0.31 ± 0.56 nmole/g, and increased FH_4 and FH_4 levels as well.

DISCUSSION

In the present study we have found that (d,l)-5-CHO-FH_4 retards tumour growth in rats (Fig. 1). The pharmacokinetics of (l)-5-CHO-FH_4 and (d)-5-CHO-FH_4 differ completely, with β $t_{1/2}$ of 7.5 hours and 32 minutes,

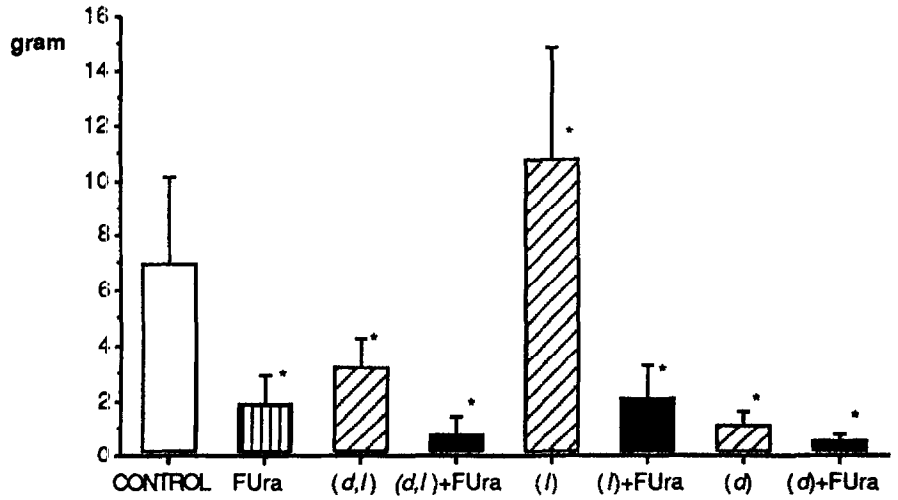

Fig. 1. Tumor weight at day seventeen in the eight groups after termination of drug treatment (means ± SD). *indicates statistically significant difference ($P < 0.05$) compared to the control group. (d,l) = (d,l)-5-CHO-FH_4 (15 mg/kg). (l) = (l)-5-CHO-FH_4 (7.5 mg/kg). (d) = (d)-5-CHO-FH_4 (7.5 mg/kg). White bars—control, striped bar—5-fluorouracil (FUra, 30 mg/kg), hatched bars—folate only, black bars—folate in combination with FUra (30 mg/kg).

Table 3. Tissue levels (means ± SD) of TS, CH_2FH_4, FH_4, and total concentrations of reduced folates in tumour tissue at day 17, 24 hours after last drug injection. All comparisons were made to control rats, and * indicates statistical significance ($P \leq 0.05$).

Treatment	TS pmole/g	Folates nmole/g		
		CH_2FH_4	FH_4	$[CH_2FH_4 + FH_4]$
Control	16.8 ± 6.14	0.38 ± 0.60	1.34 ± 1.33	1.72 ± 1.88
FUra	12.5 ± 4.66	2.06 ± 2.82	2.27 ± 1.25	4.33 ± 3.85
(d/l)-5-CHO-FH_4	13.2 ± 2.35	0.84 ± 1.11	1.72 ± 0.85	2.57 ± 2.06
FUra + (d/l)-5-CHO-FH_4	10.5 ± 4.04	0.75 ± 0.85	1.57 ± 1.48	2.31 ± 2.38
(l)-5-CHO-FH_4	27.5 ± 8.36*	0.48 ± 0.79	2.26 ± 1.05	2.74 ± 1.42
FUra + (l)-5-CHO-FH_4	10.1 ± 5.95	1.07 ± 0.89	2.01 ± 0.94	2.91 ± 1.77
(d)-5-CHO-FH_4	18.0 ± 5.07	1.07 ± 1.23	1.87 ± 0.60	2.94 ± 1.72
FUra + (d)-5-CHO-FH_4	12.2 ± 5.29	5.64 ± 5.50*	1.88 ± 0.85	7.52 ± 5.58*

respectively, in human plasma for the two isomers [22]. Significant differences in pharmacokinetics of the two isomers have also been reported in animals [23]. Thus, repeated exposure will result in increasing plasma levels of the unnatural isomer. (d)-5-CHO-FH_4 is less efficiently transported into cells than (l)-5-CHO-FH_4, but it has been shown that (d)-5-CHO-FH_4 acts as a competitive inhibitor of (l)-5-CHO-FH_4 transport [24].

The mechanisms of the direct antitumour effect of (d,l)-5-CHO-FH_4 fall outside the scope of this study. Although (d,l)-5-CHO-FH_4 has been widely used clinically for more than 30 years, its metabolic role is not yet completely known. Recent studies by Stover and Schirch [25] indicate that naturally occurring (l)-5-CHO-FH_4 is the result of conversion of 5,10-methenyltetrahydrofolate by serine hydroxymethyltransferase (SHMT, EC 2.1.2.1). In turn, (l)-5-CHO-FH_4 is a slow tight binding inhibitor of the latter enzyme. The active folate interacting with TS and FdUMP is CH_2FH_4, and the currently accepted theory for 5-CHO-FH_4 enhancement of FUra treatment efficacy is expansion of intracellular CH_2FH_4 and/or FH_4 pools [26, 15]. In mice, CH_2FH_4 and pools are elevated 250 to 400%, which supports this hypothesis, but elevation of the reduced folate pool does not account for the inhibition of tumour growth in animals not treated with FUra. However, the expanded folate pools decline rapidly, and approach control levels six hours after 5-formyltetrahydrofolate injection [23].

(l)-5-CHO-FH_4 comprises 30% of the intracellular folate pool, and is an important inhibitor of many folate dependent enzymes. A complex enzyme system exists intracellularly for interconversion of folates and the formation of CH_2FH_4 from exogenous (d,l)-5-CHO-FH_4, but it is still not known if ((d)-5-CHO-FH_4 and (l)-5-5-CHO-FH_4 are metabolised to CH_2FH_4 with the same efficiency. Polyglutamates of (d,l)-5-CHO-FH_4 have been reported to inhibit several enzymes involved in one-carbon donor metabolism, and these inhibitory effects are restricted to the polyglutamates. 5,10-methenyltetrahydrofolate synthase (EC 6.3.3.2), which has been isolated from human liver [27], is the only known enzyme that uses (l)-5-CHO-FH_4 as a substrate. 5,10-methenyltetrahydrofolate synthase is strictly stereoselective across species, and there is no basis to suppose that (d)-5-CHO-FH_4 could be metabolised intracellularly. Using MCF-7 human breast cancer cells, Bertrand and Jolivet found that inhibition of methenyltetrahydrofolate synthetase by 5-formyltetrahydrohomofolate resulted in a two-fold increase in cellular (d,l)-5-CHO-FH_4 concentration with a concomitant and equal decrease in cellular 5,10-methenytetrahydrofolate concentration. It was also found that the increased cellular levels of (d,l)-5-CHO-FH_4 were correlated with decreases in both cell growth and rate of *de novo* purine biosynthesis [28].

It was further reported that (l)-5-CHO-FH_4 inhibits *E. coli* TS noncompetitively versus CH_2FH_4 [29, 30]. Although (d)-5-CHO-FH_4 is a considerably less potent TS inhibitor compared to (l)-5-CHO-FH_4 + FUra *in vitro*, it may influence natural folate interactions with TS, since the inhibitory effect of (d,l)-5-CHO-FH_4 is five times lower compared to (l)-5-CHO-FH_4 [13].

(l)-5-CHO-FH_4 has shown itself to be an effective tumour growth stimulator in this study. This growth stimulatory effect was also associated with a significant increase in intratumour TS activity. TS levels were measured 24 hours after the last 5-formyltetrahydrofolate injection, and only (l)-5-CHO-FH_4 was associated with a significant increase in all eight treatment groups. One reasonable explanation for this could be an induction of TS gene expression [10, 13], but decreased TS catabolism potentially could occur. Increases in TS and (l)-CH_2FH_4 after (l)-5-CHO-FH_4 might be expected, *a priori*, to be associated with increased growth rate.

In this study the most pronounced tumour growth inhibition was surprisingly found after administration of (d)-5-CHO-FH_4 and (d)-5-CHO-FH_4 in combination with FUra. Only limited data are available regarding the effects of (d)-folates on folate dependent enzymes, and since the intracellular metabolism of (d)-5-CHO-FH_4 is unknown, the biochemical rationale for this remains to be explained. The antitumour effect of (d,l)-5-CHO-FH_4 may be largely due to the unnatural (d)-5-CHO-FH_4, and it may be hypothesized that the enhancement of FUra cytotoxicity is not a potentiation but an additive effect of (d)-5-CHO-FH_4, since (l)-5-CHO-FH_4 did not significantly enhance the FUra-effect nor expand the folate pools in this study. In a previous report by O'Connell, it was suggested that low-dose (d,l)-5-CHO-FH_4 (20 mg/m^2) results in higher response rates than a higher dose (200 mg/m^2) in combination with FUra [31]. One explanation for this could be that the cytotoxic effects of (d)-5-CHO-FH_4 predominate at lower concentrations, whereas the tumour growth stimulatory effects of (l)-5-CHO-FH_4 dominate at higher concentrations. We have recently found, when studying the effects of the different diastereoisomers of CH_2FH_4 in a rat colon adenocarcinoma, that unnatural (d)-CH_2FH_4 seems to be an inhibitor of tumour growth *per se*, whereas modulation of FUra activity is the main effect of (l)-CH_2FH_4, which by itself acts as a tumour promoter [32].

In this study we have reported results *in vivo* showing that the biological effects of the two different diastereo-

isomers of 5-CHO-FH_4 seem to have completely opposite effects as single agents. Treatment with unnatural (d)-5-CHO-FH_4 alone was associated with slower tumour growth, whereas natural (l)-5-CHO-FH_4 promoted tumour growth.

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