Cancer Chemother Pharmacol (2000) 45: 165–171

ORIGINAL ARTICLE

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Impact of the simultaneous administration of the (+)- and (−)-forms of formyl-tetrahydrofolic acid on plasma and intracellular pharmacokinetics of (−)-tetrahydrofolic acid

Received: 12 March 1999 / Accepted: 9 August 1999

Abstract *Purpose*: To detect possible interactions between (−)-formyl-tetrahydrofolic acid (leucovorin, (−)-fTHF) and (+)-formyl-tetrahydrofolic acid ((+)-fTHF) on the plasma and intracellular pharmacokinetics following their simultaneous administration. *Methods*: Plasma levels of (−)-fTHF, (−)-methyl-THF, and (+)-fTHF were determined in samples from four volunteers following the administration of both (−)-fTHF and (±)-fTHF and in seven patients during a 5-fluorouracil (5-FU)/fTHF combination chemotherapy. In addition, the intracellular uptake of ^{14}C-(−)-mTHF in the presence of (+)-mTHF at increasing concentrations was measured in vitro. Analyses were performed using a highly specific high-performance liquid chromatography procedure. *Results*: The pharmacokinetic parameters obtained for (−)-fTHF following the administration of (−)-fTHF only were: terminal half-life, 1.2 h; area under the curve, 10 μg · h/ml; maximum concentration, 12 μg/ml; clearance, 305 ml/min; volume of distribution, 19 l. The parameters did not differ significantly as compared with those obtained following the administration of (±)-fTHF to both volunteers and patients. There were no differences in the pharmacokinetics of (−)-mTHF or in the protein binding of both substances with the different forms of administration. The intracellular uptake of ^{14}C-(−)-mTHF did not depend on the presence of (+)-mTHF at either concentration. *Conclusions*: These data suggest that (−)-fTHF is not therapeutically superior to (±)-fTHF and that the latter is appropriate during combination chemotherapy with 5-FU/fTHF in patients with colorectal cancers.

Key words Colorectal cancer · Diastereoisomers · Leucovorin · Pharmacokinetics

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Introduction

Colorectal cancer is the fourth most frequent malignancy in most Western countries with an incidence of 50:100,000 [51]. While surgery is the treatment of choice for limited stages of the disease, more advanced stages are treated with palliative intention usually applying the standard combination of 5-fluorouracil (5-FU) and folinic acid (leucovorin, formyl-tetrahydrofolic acid, fTHF) [19, 28, 39]. In numerous randomized clinical trials, the addition of fTHF to 5-FU has been shown to significantly improve the antitumor activity and to prolong the survival of patients receiving adjuvant chemotherapy or treatment for advanced disease as compared to monotherapy with 5-FU alone [9, 34, 35, 49, 52]. Furthermore, in patients with tumor progression during therapy with 5-FU alone the administration of the fTHF/5-FU-combination results in responses in some patients [4, 8, 20, 21, 23, 49]. Formyl-THF comprises a racemate of its (−)- and (+)-forms. When it was found that only (−)-fTHF is biologically active [3, 21, 26, 29, 30, 42, 46, 48] it appeared appropriate to administer the (−)-form only in order to avoid a possible impairment of (−)-fTHF activity by (+)-fTHF which is not metabolized in humans and is slowly eliminated in the urine as unchanged compound. Along this line, it was suggested that (+)-fTHF interferes with the metabolism and the elimination of (−)-fTHF as well as with its binding to plasma proteins. Furthermore, (+)-fTHF could potentially decrease the intracellular accumulation of (−)-fTHF by competitive inhibition of the transmembrane folate carrier. To further clarify the effect of the administration of (+)-fTHF on the pharmacology of (−)-fTHF during

treatment with racemic fTHF, pharmacokinetic analyses of (+)-fTHF and (−)-fTHF during the administration of both (−)-fTHF and (±)-fTHF were performed by high-performance liquid chromatography (HPLC). This method might be more convenient than the formerly used microbiological assays and also shows comparable sensitivity and specificity [21, 25, 26, 30, 40, 42, 46, 48]. In addition, the influence of (+)-methyl-THF ((+)-mTHF) on the cellular uptake of ^{14}C-(−)-mTHF was assessed in vitro.

Healthy volunteers, patients, and methods

Healthy volunteers and drug administration

Four healthy volunteers received 10-min infusions of the racemate (±)-fTHF at a dose level of 200 mg/m^2. This was followed by the administration of the diastereoisomer (−)-fTHF as a 10-min infusion at a dose level of 100 mg/m^2 1 week later.

Patients and chemotherapy

Patients with normal renal and hepatic function receiving combination chemotherapy with 5-FU and fTHF for advanced colorectal cancers were eligible for the current study. The racemate (±)-fTHF was administered as a 15-min infusion at a dose level of 300 mg/m^2 followed by a short-term infusion of 5-FU at a dose of 425 mg/m^2.

Acquisition of blood and urine samples

Venous blood samples were drawn from volunteers and patients before the infusion of fTHF and at 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 10, 12, and 24 h after its end. The plasma was separated immediately. Urine samples were taken from volunteers before the infusion and 4, 8, 12, and 24 h after its end. To all samples 1 mg/ml ascorbate was added to prevent degradation of the drug. The samples were stored immediately at −20 °C until analysis for a maximum of 1 week.

Study conduct

Prior to therapy all volunteers and patients gave their informed consent for participation in the current evaluation after having been advised about the purpose and investigational nature of the study as well as of the potential risks. The study design adhered to the Declaration of Helsinki and was approved by the local ethics committee prior to its initiation.

Sample preparation

The blood samples were prepared as described previously [44]. In brief, after the addition of ascorbic acid (0.5 mg/ml) for stabilization and of acetonitrile (ratio 1:1.5 v/v, plasma/acetonitrile), vortex mixing, and centrifugation at 400 *g* for 1 min, 7 ml chloroform was added to the supernatant of all plasma samples to extract the acetonitrile. After a second mixing and centrifugation at 400 *g* for 1 min, a maximum of 400 ml of the aqueous phase of the resulting solution was diluted with eluent A (ratio 1:5 v/v).

For urine samples with folate concentrations above 500 ng/ml, 50 µl urine was mixed with 500 µl eluent A and directly injected into the HPLC system. Other samples were diluted with eluent A (1:4) and enriched on 1-ml Sulpelco C18 solid-phase extraction cartridges followed by elution of matrix substances with 2 ml eluent A. Folates were then eluted with 2 ml 50% acetonitrile with the subsequent preparation being identical to that for plasma samples. The preparation procedure of urine samples was only necessary for folate concentrations less then 500 ng/ml.

HPLC of (+)- and (−)-mTHF and (−)-fTHF

Plasma folates were separated stereoselectively using a highly sensitive achiral-chiral HPLC system [44]. Following enrichment of the folates on a 5-µm octadecyl silica material cartridge using eluent A (0.005 *M* TBAP, low-UV, Waters) adjusted to pH 6.5 with phosphoric acid, the samples were separated within the achiral part of the system on a 3-µm C18 column (eluent B, 0.0015 *M* sodium phosphate, 0.00075 *M* TBAP, 7.5% v/v 2-propanol) adjusted to pH 5 with phosphoric acid. A BSA-7 column was used for the chiral part of the system after flushing with eluent C (0.028 *M* phosphate buffer, 0.0006 *M* sodium azide). Since mTHF showed a pH-dependent reciprocal fluorescence emission, water adjusted to pH 1.5 with phosphoric acid (eluent D) was mixed with the flow of eluent B by a reagent dosing pump. The detection level was 5 ng/ml for each stereoisomer of fTHF and for (−)-mTHF.

Assessment of the binding of folates to plasma proteins

Folate levels of plasma samples obtained from patients 2 h after the infusion of 300 mg/m^2 (±)-fTHF were determined by HPLC directly after sample preparation as well as after ultrafiltration.

The binding of folates to plasma proteins of samples from healthy volunteers was assessed in vitro. Following the addition of aqueous solutions of different diastereomeric folates at defined concentrations to plasma samples, the binding of folates to plasma proteins was measured as described above. The binding to plasma proteins was assessed following the addition of equal doses of (−)-fTHF, (−)-mTHF, and (+)-fTHF to the plasma samples as compared with the addition of only (−)-fTHF and (−)-mTHF. Furthermore, the binding of (+)-fTHF, (−)-fTHF, and (−)-mTHF was quantified for increasing concentrations of all three compounds. All experiments were performed in triplicate.

Pharmacokinetic evaluations

Analysis of the pharmacokinetic results was based on the TOPFIT computer program providing an optimized adaptation of coefficients of variation between the observed and calculated data [14]. Data for (−)-fTHF and (−)-mTHF were calculated simultaneously, with the rate of metabolism of (−)-fTHF assumed to be first-order. For the calculation of the dose-dependent parameters of (−)-mTHF the fictitious applied dose of (−)-mTHF was set equal to the amount of (−)-mTHF detected in the urine, assuming a nearly complete renal elimination of this compound.

In vitro determinations of cellular folate uptake

The cellular uptake of ^{14}C-(−)-mTHF at increasing concentrations of (+)-mTHF was analyzed in Raji (Burkitt lymphoma), HL60 (acute myeloid leukemia), and K562 (chronic myelogenous leukemia) cell lines. Incubations were performed for 3 h at a concentration of 1 µg/ml for ^{14}C-(−)-mTHF and concentrations of 0, 5, 10, 20, 50, and 100 µg/ml for (+)-mTHF. After centrifugation and lysis of the cells the radioactivity was assessed in a liquid scintillation counter using external standards. All experiments were carried out in triplicate.

Statistics

All experiments on the binding of folates to plasma proteins in volunteers as well as all in vitro experiments were carried out in triplicate. The results are given as mean values and coefficients of variation of the three determinations. Pharmacokinetic data were calculated separately for each individual. Mean values and coeffi-

cients of variation for each parameter were then calculated and are provided for both volunteers and patients.

Comparisons of mean values were performed using Student's *t*-test. To account for differences in the administered doses of fTHF, the dose-dependent pharmacokinetic parameters obtained for volunteers were multiplied 1.5-fold for the respective comparisons to values of patients. Relationships between different concentrations of folates and their binding to plasma proteins and the cellular uptake of (−)-mTHF were assessed using the Spearman rank-test.

Results

Patients and healthy volunteers

Seven patients (five male, median age 54 years, range 45–65 years) and four healthy volunteers (three male, median age 34 years, range 31–38 years) were included in the current study, all of whom were given folate as described above.

Pharmacokinetics in healthy volunteers

The fitting of the respective measurements to a two-compartment model resulted in the pharmacokinetic parameters shown in Table 1. The AUC as well as all other parameters were apparently identical for both forms of administration. There was no indication of an effect of (+)-fTHF on the pharmacokinetics of (−)-fTHF. Accordingly, in all volunteers the courses of the plasma levels of (−)-fTHF and (−)-mTHF were congruent for both forms of administration. Figure 1 shows example data obtained from one volunteer's samples. Following the maximum plasma level at the end of the infusion, the decay of (−)-fTHF levels occurred much faster than the decay of (+)-fTHF levels ($t_{1/2\beta}$ 1.2 h versus 6.7 h), and 10 h after the infusion (−)-fTHF was no longer detectable. The maximum concentration of (−)-mTHF was reached at 2.5 h and declined thereafter with a terminal half-life of 4 h.

The amounts of (−)-fTHF, (−)-mTHF, and (+)-fTHF detected in the urine were 33% (coefficient of variation 21%), 57% (18%), and 69% (34%) following administration of the racemate and 34% (8%), 57% (12%), and 0% following administration of (−)-fTHF (Student's *t*-test, not significant).

Pharmacokinetics in patients

To verify the analyses of the samples from volunteers, pharmacokinetic evaluations were carried out on samples obtained from patients during the administration of (±)-fTHF. The fitting of the respective measurements to a two-compartment-model resulted in the pharmacokinetic parameters shown in Table 1. The data for the dose-independent parameters were almost identical to those obtained from the volunteers' samples, while the slightly higher values for AUC and maximum concentration were due to the dose increase to 150%. Accordingly, the courses of the plasma levels revealed the same characteristics as described for the volunteers (Fig. 2). After 10 h, (−)-fTHF was not detectable, and the concentrations of (+)-fTHF declined more slowly than those of (−)-mTHF ($t_{1/2\beta}$ 6.6 h versus 3.3 h).

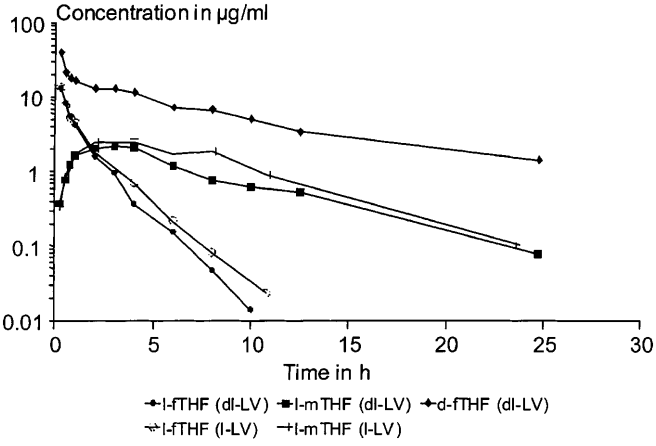

Fig. 1 Time-courses of the levels of (−)-fTHF, (−)-mTHF, and (+)-fTHF in plasma from a volunteer following the administration of both (±)-fTHF and (−)-fTHF

Table 1 Pharmacokinetic parameters of fTHF and metabolites in volunteers following the administration of (±)-fTHF and (−)-fTHF and in patients following the administration of (±)-fTHF ($t_{1/2\beta}$ terminal half-life, *AUC* area under curve, C_{max} maximum concentration, t_{max} time-point of maximum concentration, Cl_{total} total clearance, V_{ss} volume of distribution during steady state). Values are means and coefficients of variation

	Volunteers					Patients		
Substances administered	(±)-fTHF			(−)-fTHF		(±)-fTHF		
Substances evaluated	(−)-fTHF	(−)-mTHF	(+)-fTHF	(−)-fTHF	(−)-mTHF	(−)-fTHF	(−)-mTHF	(+)-fTHF
$t_{1/2\beta}$ (h)	1.2 (26%)	4.1 (36%)	6.7 (17%)	1.15 (14%)	3.9 (27%)	0.76 (12%)	3.3 (30%)	6.6 (22%)
AUC (µg · h/ml)	10 (21%)	19 (29%)	134 (14%)	11 (22%)	20 (20%)	15 (32%)	31 (44%)	251 (42%)
C_{max} (µg/ml)	12 (29%)	2.5 (24%)	24 (34%)	12 (16%)	2.5 (15%)	26 (57%)	3.8 (48%)	51 (44%)
t_{max} (h)	–	2.2 (17%)	–	–	2.5 (16%)	–	2.4 (18%)	–
Cl_{total} (ml/min)	305 (27%)	110 (26%)	23 (20%)	285 (28%)	98 (22%)	339 (42%)	–	20 (38%)
V_{ss} (l)	19 (18%)	32 (34%)	12 (30%)	19 (12%)	29 (24%)	18 (47%)	–	11 (36%)

There were no significant differences between the values for any of the individual substances evaluated

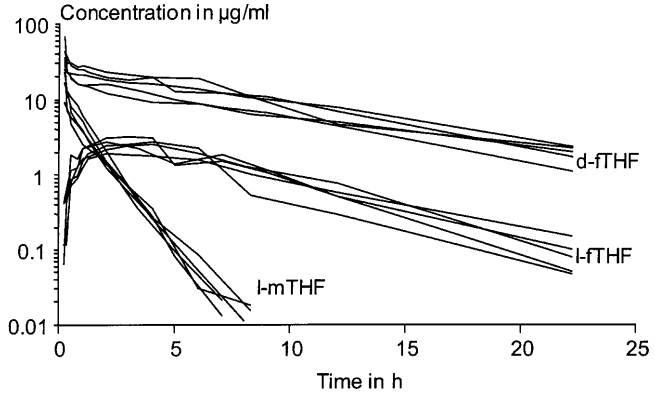

Fig. 2 Time-courses of the levels of (−)-fTHF, (−)-mTHF, and (+)-fTHF in plasma from patients following the administration of (±)-fTHF

The amounts of (−)-fTHF, (−)-mTHF, and (+)-fTHF detected in the urine were 33% (coefficient of variation 21%), 57% (18%), and 69% (34%) following administration of the racemate and 34% (8%), 57% (12%), and 0% following administration of (−)-fTHF (Student's *t*-test, not significant).

In vitro binding of folates to plasma proteins in samples from volunteers

The binding of (−)-fTHF, (−)-mTHF, and (+)-fTHF to plasma proteins following the incubation of volunteers' plasma samples with all three substances as well as with solely the (−)-forms under the same conditions at 2 µg/ml is shown in Table 2. The binding of the (−)-forms to plasma proteins was not influenced by (+)-fTHF. A saturation or a competitive displacement was not observed. In addition, throughout the range 0.1 to 100 µg/ml at increasing concentrations of each substance a displacement from the binding to plasma protein was excluded (Table 3).

In vivo binding of folates to plasma proteins in patients

The binding of folates to plasma proteins in patients in vivo following the administration of 300 mg/m^2 (±)-fTHF was identical to the results in volunteers (Table 2).

Table 2 Binding of (−)-fTHF, (−)-mTHF, and (+)-fTHF to plasma proteins in samples from volunteers in vitro and in patients in vivo (means and coefficients of variation)

	Samples from volunteers, in vitro Incubation using		Patients, in vivo
	(−)-fTHF, (−)-mTHF, (+)-fTHF	(−)-fTHF, (−)-mTHF	
(−)-fTHF	32%* (8%)	27% (3%)	25* (33%)
(−)-mTHF	59%* (6%)	73% (4%)	57* (18%)
(+)-fTHF	88%* (11%)	–	88* (8%)

* Student's t-test, not significantly different

Table 3 Binding of (−)-fTHF, (−)-mTHF, and (+)-fTHF to plasma proteins in samples from volunteers in vitro

Concentration (µg/ml)	Binding to plasma proteins (%)			
	(−)-fTHF*	(−)-mTHF*	(+)-fTHF*	(+)-mTHF*
100	36	66	83	86
10	41	60	87	83
1	37	62	85	84
0.5	39	59	83	83
0.25	40	61	91	90
0.1	31	59	83	90
Mean	37	61	85	86
Coefficient of variation	4%	3%	3%	3%

* Spearman rank-test for correlation with concentration: not significant

In vitro examinations of cellular folate uptake

Following incubations at increasing concentrations of (+)-mTHF for 3 h the intracellular accumulation of ^{14}C-(−)-mTHF did not change in any of the three cell lines tested (Table 4).

Discussion

In the current study the potential impact of (+)-THF on the pharmacokinetics of racemic fTHF in patients receiving combination chemotherapy for colorectal cancers was analyzed. The analyses of samples from volunteers and patients revealed no influence of (+)-fTHF on the metabolization and elimination or on the binding to plasma proteins of (−)-fTHF. In addition, an inhibition of the intracellular uptake of (−)-mTHF due to competition by (+)-mTHF for the transmembrane folate carrier was ruled out.

Following the observation that modulation of fluorouracil by the administration of fTHF significantly improves both the response rate [1, 9, 17, 32, 34, 35] and the survival [10, 33, 36, 37] in patients receiving these agents for advanced colorectal cancers, the combination of fTHF and 5-FU has become standard therapy in this group of patients. Furthermore, high-dose fTHF in

Table 4 Intracellular acumulation of ^{14}C-(−)-mTHF at increasing concentrations of (+)-mTHF. Values are means and coefficients of variation

(+)-mTHF (µg/ml)	(−)-mTHF (ng per 2.5 × 10^6 cells)		
	Raji*	HL60*	K562*
0	11.9 (10%)	15.7 (6%)	14.5 (16%)
5	12.9 (9%)	15.8 (5%)	15.0 (16%)
10	12.8 (6%)	15.7 (5%)	15.5 (13%)
25	12.4 (9%)	15.3 (4%)	13.9 (14%)
50	12.8 (12%)	15.3 (3%)	15.4 (3%)
100	12.2 (14%)	15.4 (9%)	15.4 (12%)

* Spearman rank-test for correlation with concentration: not significant

addition to 5-FU given as adjuvant therapy has been shown to significantly prolong the survival of patients with colorectal cancers as compared to surgery alone and its use as standard therapy has been extended also to patients with operable colorectal carcinomas [52]. Based on these results, a variety of studies have addressed the question as to whether the dose of fTHF could be decreased without loss of antitumor efficacy, and have clearly demonstrated that low-dose fTHF [5, 15, 16] or (−)-fTHF [18] is equally effective in patients with advanced colorectal cancers to a high-dose schedule.

Focusing on another aspect of the mechanisms of action of fTHF, efforts to improve the efficacy of the fTHF/5-FU combination have been made by administering only the biologically active (−)-stereoisomer instead of (±)-fTHF in order to prevent a pharmacologically based impairment of the intracellular activity of (−)-THF by the biologically inactive (+)-THF. Along this line, combinations of (−)-fTHF and 5-FU have been administered to patients with colorectal cancers [22, 38] and breast cancers [53] in phase II trials, and have shown substantial activity. Furthermore, (−)-fTHF has been shown to be effective as rescue therapy during high-dose methotrexate treatment in children with acute lymphoblastic leukemia [11].

To further delineate possible interactions between (−)-THF and (+)-THF, in the current study for the first time the pharmacokinetics of both substances following the administration of the racemate were determined as well as those of (−)-THF after administration of this compound only to healthy volunteers. The design of this study, comprising a crossover for each individual, guaranteed an adequate analysis of any drug interaction. In addition, the high sensitivity of the method applied (limit of detection 5 ng/ml) allowed an accurate assessment of the terminal half-lives of all compounds. On this basis no differences in evaluated pharmacokinetic parameters were detected between the two forms of administration. Hence, the terminal half-lives for (−)-fTHF and (−)-mTHF were 1.2 versus 1.15 h and 4.1 versus 3.9 h, respectively. Also, the AUCs (10 versus 11 μg h/ml and 19 versus 20 μg · h/ml), the clearances (305 versus 285 ml/min and 110 versus 98 ml/min), and volumes of distribution (19 versus 19 l and 32 versus 29 l) did not differ significantly.

The pharmacokinetic data obtained for patients in the current study receiving the racemate were almost identical to the parameters in volunteers and fitted well within the range of previously described data, which were also obtained using stereoselective HPLC methods to assess the pharmacokinetics of both isomers [24, 27, 30, 40, 42, 43, 45–48, 54]. In contrast, analyses based on non-stereoselective procedures have revealed less conclusive results [2, 7, 13, 23, 25, 26]. Furthermore, the assessment of the protein binding of either substance in vivo as well as in vitro did not indicate any effect of (+)-THF on (−)-THF. These results have in part been confirmed in other in vivo studies [31] and argue – with regard to the pharmacokinetic data outlined above – for a stereoselectiveness of the protein binding [31] as well as of the absorption of folates following oral administration [26, 42]. Thus, the administration of (+)-THF does not seem to affect the pharmacology of (−)-THF.

However, the results of in vitro studies in L1210 murine leukemia cells suggest an interaction of (+)-THF and (−)-THF on the cellular level resulting in a competitive inhibition of the intracellular uptake of both compounds [6]. This could not be confirmed in the current analyses of the cellular uptake of ^{14}C-(−)-mTHF, which was not inhibited in the presence of (+)-mTHF within a wide range of concentrations. (+)-mTHF was chosen for the inhibition experiments since this compound can be separated completely from the respective (−)-form which is not the case for (+)-fTHF. Furthermore, selectivity of folate carriers has been described for stereoisomers but not for m- or f-THF [50]. Thus, the current analyses covered the equivalent of maximum concentrations of (+)-fTHF observed in vivo. Accordingly, other studies also addressing the influence of (+)-mTHF on the intracellular uptake of (−)-mTHF could not detect any interaction of the two compounds at any concentration [3, 6, 43].

Overall, the administration of (±)-THF resulted in identical plasma and cellular pharamacokinetics to the administration of (−)-THF only. The present results have been confirmed in clinical trials which have failed to reveal any superiority of (−)-THF in combination with 5-FU as compared with the (±)-THF/5-FU combination in terms of tumor response and survival in patients with advanced colorectal cancers [12, 41].

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