Red Blood Cell Partitioning of the [6 *S*]- and the [6 *R*]-Isomer of N_5-Formyltetrahydrofolic Acid

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Z. Naturforsch. **47 c,** 748–752 (1992); received March 16/July 16, 1992

N_5-Formyltetrahydrofolic Acid, [6 *S*]- and [6 *R*]-Stereoisomers, Red Blood Cell Coefficient of Partition

The *in vitro* interaction of the [6 *S*]- and [6 *R*]-stereoisomers of CHO-THFA with human RBCs was investigated in the (therapeutically comparable) concentration range from 1.0 to 12.5 µg/ml. Both compounds are bound to RBCs with a k_{RBC} ranging from 0.13 to 0.75 for [6 *S*]-CHO-THFA and from 0.06 to 0.33 for [6 *R*]-CHO-THFA, respectively. The interaction of the [6 *S*]-form with RBCs is about two times higher than of the [6 *R*]-form. Incubation of CHO-THFA with RBCs over 24 h showed an accelerated disappearance from the test solution for [6 *R*]-CHO-THFA with a mean $t_{1/2}$ of 49.9 h in compare to $t_{1/2}$ = 58.2 h for the [6 *S*]-enantiomer. The results indicate that RBCs may play a major role for the pharmacokinetics and metabolism of CHO-THFA and may act as an intravasal depot especially for [6 *S*]-CHO-THFA.

The high dose administration of the racemic folinic acid ([6 *RS*]-N_5-formyltetrahydrofolic acid, CHO-THFA) combined with the antimetabolite 5-fluorouracil (5 FU) has been found to improve the tumoral response of 5 FU in the treatment of colonadenocarcinoma [1, 2] or breast cancer [3]. The way of action of CHO-THFA might be a "biomodulation" by forming a stable ternary covalent complex with 5 FU and the active enzyme thymidilat synthetase [4]. The biochemical rationale for this approach presumes, that high doses of folinic acid increase the cellular pools of reduced folates [5]. Conversion of CHO-THFA to N_5,N_{10}-methylenetetrahydrofolate (CH_2-THFA) enhances the formation and stability of the ternary FdUMP – thymidilate synthetase – CH_2-THFA complex, which in turn causes a profound inhibition of dTMP *de novo* synthesis and consequently results in impairment of DNA synthesis [6–8].

CHO-THFA has two chiral carbon centers: one chiral center is located on the 6-position in the pteridine ring portion and the other is in the terminal glutamate portion of the molecule (Fig. 1). Thus, CHO-THFA has four different optical isomers. Commercially available CHO-THFA, however, is a 1 : 1 mixture of [6 *R*]- and [6 *S*]-isomers as only l-glutamate is used in the synthetic process.

The pharmacokinetic behaviour of the two isomers differs in that the [6 *S*]-isomer is selectively absorbed from the gastroenteric tract and has a

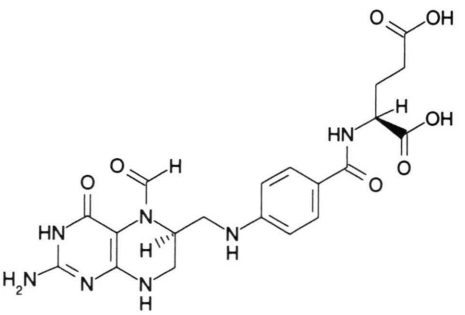

Fig. 1. Chemical structure of CHO-THFA.

Abbreviations: RBCs, red blood cells; k_{RBC}, red blood cell coefficient of partition; CHO-THFA, formyltetrahydrofolic acid; CH_3-THFA, methyltetrahydrofolic acid; NaCl, isotonic sodium chloride solution.

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0939–5075/92/0900–0748 $ 01.30/0

shorter half-life [8]. Thus, following intravenous administration, the [6*R*]-isomer tends to accumulate in the circulation relative to [6*S*]-CHO-THFA. Although there exists a lot of information about the pharmacokinetics of the [6*S*]-CHO-THFA and [6*R*]-CHO-THFA [1, 8, 10–12], nothing is known about the interaction of the stereoisomers with RBCs.

In a previous *in vivo* study we found out that the racemic CHO-THFA is bound from 12 to 40% to RBCs [9]; however, as the therapeutically active form of CHO-THFA is the [6*S*]-enantiomer, the purpose of the present study was to find out, whether there is a difference in the binding rate of [6*S*]- and [6*R*]-CHO-THFA to RBCs or not.

Materials and Methods

Chemicals

All solvents were from HPLC grade purity (Merck, Darmstadt, Germany). Folinic acid ([6*RS*]-folinic acid, Calcium Folinat) was obtained from Ebewe (Unterach, Austria), [6*S*]-folinic acid, L-Leucovorin 200 was donated from Lederle-Cyanamid (Wolfratshausen, Germany) as an aqueous solution containing 200 mg of the drug in 20 ml solution for injection. Isotonic and sterile sodium chloride solution was purchased from Heilmittelwerke (Vienna, Austria).

Red blood cells purification

RBCs were obtained as a fresh "erythrocyte concentrate" from the surgical department of the Hospital-Rudolf-Stiftung. The RBCs were washed with isotonic sodium chloride solution as described [13, 14], centrifuged for 5 min at 6000 rpm (Biofuge B, Haereus Christ, Vienna, Austria) and finally diluted with isotonic sodium chloride solution for a hematocrit of 0.45. These solutions were used immediately for the experiments.

Drug solutions and binding experiments

CHO-THFA was directly used from the manufacturer's formulations, diluted with isotonic sodium chloride solution and pipetted into 1.0 ml of the fresh prepared RBC suspension to give final concentrations from 1.0 to 12.5 μg/ml (for concentrations see Tables I and II). The samples were vortexed for 1 min (Paramix II, Julabo, Seelbach, Germany) and kept in dark at 37 °C for 20 min (to avoid photodegradation of the drug). The suspension was centrifuged at 6000 rpm for 4 min (Biofuge B, Haereus Christ, Vienna, Austria), the supernatant was filtered through a 20 μm filter (Millipore, Bedford, U.S.A.) and injected directly into the liquid chromatograph without further purification. For each concentration the binding experiments were performed four times to obtain minimal statistical information. As a control each set of experiments was performed with isotonic sodium chloride solution, too.

Analytical procedure

Quantitation of [6*S*]- and [6*R*]-CHO-THFA was performed by high performance liquid chromatography using a chiral BSA column as described [15–17].

Biometric calculations

The red blood cell coefficient of partition (k_{RBC}) was calculated as given in equation (1), whereby c_{RBC} is the concentration of CHO-THFA found in the RBCs and c_{SN} is the concentration in the supernatant. The percentual amount of CHO-THFA in the RBCs ($\%_{RBC}$) was calculated as given in equation (2).

$$k_{RBC} = \frac{c_{RBC}}{c_{SN}} \tag{1}$$

$$\%_{RBC} = \frac{c_{RBC} \times 100}{c_{RBC} + c_{SN}} \tag{2}$$

The area under the concentration time curves (AUC_{0-24}) were calculated *via* the trapezoidal rule.

The degradation half-life of the compounds was calculated by use of the program "PCNonlin" on a PC AT 386 SX 20, Anova analysis of variance was performed by use of the scientific package "Statist" on an Atari MegaST. Multiple iterative regression analysis of CHO-THFA concentrations *versus* c_{SN}, c_{RBC} and k_{RBC} was performed on an Atari MegaST using the program Wistat. Conversion and plotting of the data in regard to binding sites was performed according a modified method of Scatchard [18].

Results and Discussion

The bound amounts of [6*S*]- and [6*R*]-CHO-THFA at different concentrations are listed in the

Table I. RBC binding of [6 *S*]-CHO-THFA.

[μg/ml]	c_{free} [μg/ml]	c_{bound}	k_{RBC}	$\%_{bound}$
1.0	0.57 ± 0.11	0.43	0.75	42.9
2.2	1.17 ± 0.40	1.03	0.86	46.7
4.0	2.67 ± 0.44	1.33	0.50	33.3
5.1	3.57 ± 0.98	1.53	0.43	30.0
7.6	5.81 ± 1.12	1.79	0.31	23.5
10.0	8.54 ± 1.32	1.46	0.17	14.6
12.5	11.09 ± 2.09	1.41	0.13	11.3
correlation	0.996	0.964	0.943	0.974
std. error	0.132	0.016	0.010	10.942
p	<0.0001	<0.0019	<0.0048	<0.0009
type	lin.	parabol.	lin.	lin.

Table II. RBC binding of [6 *R*]-CHO-THFA.

[μg/ml]	c_{free} [μg/ml]	c_{bound}	k_{RBC}	$\%_{bound}$
1.0	0.75 ± 0.15	0.25	0.33	25.0
2.2	1.69 ± 0.28	0.51	0.35	23.3
4.0	3.27 ± 0.33	0.73	0.22	18.2
5.1	4.25 ± 0.88	0.85	0.20	16.7
7.6	6.74 ± 1.01	0.86	0.13	11.3
10.0	9.26 ± 1.24	0.74	0.08	7.4
12.5	11.84 ± 1.66	0.66	0.06	5.3
correlation	0.999	0.968	0.961	0.990
std. error	0.039	0.004	0.001	1.382
p	<0.0001	<0.0015	<0.002	<0.0001
type	lin.	parabol.	lin.	lin.

Tables I and II. The bound amount decreases from 40% (at 1 μg/ml) to 11% (at 12.5 μg/ml) for [6 *S*]-CHO-THFA and from 25% (at 1 μg/ml) to 5% (at 12.5 μg/ml) for the [6 *R*]-isomer.

The absolute amounts of bound CHO-THFA are directly proportional to the amount in the test solutions, but the percentual amount of bound drug decreases with increasing drug concentrations in the test solutions. This indicates, that the RBCs own a certain capacity for interaction (or incorporation) of CHO-THFA, but this capacity is limited at higher concentrations of the free drug. The close correlation between drug concentration and the binding parameters are listed in Tables I and II, respectively, whereby a linear regression can be calculated between drug concentration and c_{free}, k_{RBC} and $\%_{bound}$ and a parabolic regression between drug concentration and c_{bound}. This phenomenon could be observed for both, the [6 *S*]- and the [6 *R*]-isomer. The level of probability was for all regressions at least for $p < 0.005$ for [6 *S*]- and $p < 0.002$ for [6 *R*]-CHO-THFA. Over the whole observed concentration range the [6 *S*]-form is bound to RBCs at about two times higher (11 to 43%) than the [6 *R*]-isomer (5 to 25%).

The analysis of the data as a Scatchard transformation confirms the limited binding capacity of the RBCs (in the therapeutical concentration range) and at "physiological" RBCs concentrations (hematocrit of 0.45). At drug concentrations higher than 6 μg/ml of free [6 *S*]-CHO-THFA the binding capacity of RBCs is reduced, thus meaning the RBC-binding sites are occupated by CHO-THFA completely (compare Fig. 2). In the case of the [6 *R*]-isomer the limit is reached at ~4 μg/ml of free drug. As the drug and RBC concentrations in the present *in vitro* study are equal for the observed *in vivo* amounts, the percentual distribution of 2:1 [6 *S*] to [6 *R*] might be in a similar order of magnitude.

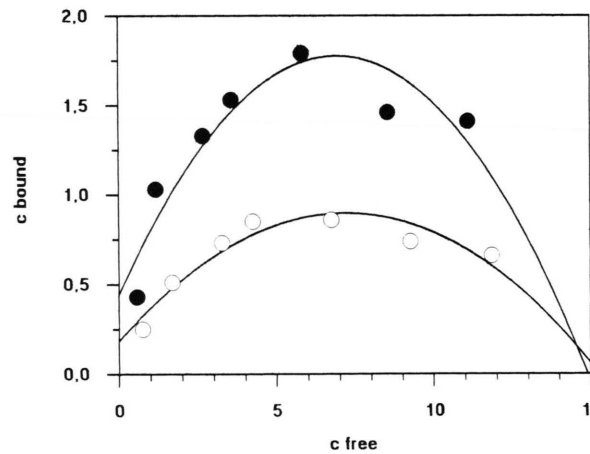

Fig. 2. Scatchard plot for [6 *S*]- (●) and [6 *R*]-CHO-THFA (○).

In a second serie of experiments, the influence of RBCs on the degradation (bioconversion) of the two isomers was studied. In the observed time interval of 24 h (at 23 °C) we found a monophasic degradation mechanism under the influence of RBCs and of NaCl solution. The mean half-lives were lower for both compounds in the RBCs ($t_{1/2}$ = 58 h for [6 *S*] and 41.9 h for [6 *R*]) than in NaCl ($t_{1/2}$ = 79.9 h for [6 *S*] and 69.9 h for [6 *R*]). The Anova analysis of variance revealed no statistical significance in the half-lives between [6 *S*] and [6 *R*] in

Table III. Kinetics of [6*S*]- and [6*R*]-CHO-THFA in RBCs and NaCl (7.6 µg/ml = 100.0%).

Time [h]	$\%_{[6S]}$RBC	$\%_{[6R]}$RBC	$\%_{[6S]}$NaCl	$\%_{[6R]}$NaCl
0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
2	91.0 ± 3.0	81.0 ± 9.5	98.0 ± 4.0	95.5 ± 8.5
4	78.5 ± 9.5	69.5 ± 7.5	96.5 ± 2.6	94.0 ± 7.9
6	78.1 ± 5.2	63.0 ± 9.8	94.5 ± 3.5	89.5 ± 7.4
12	75.5 ± 0.5	66.5 ± 8.5	84.0 ± 0.5	84.0 ± 6.9
20	73.0 ± 1.5	63.2 ± 6.6	83.5 ± 1.2	78.5 ± 3.5
24	63.1 ± 6.0	53.3 ± 4.5	79.0 ± 3.0	75.1 ± 8.8
$t_{1/2}$el	58.2	41.9	79.9	69.9
AUC_{0-24}	1852.3	1638.8	2108.2	2040.1
$\frac{AUC_{RBC}}{AUC_{NaCl}}$	0.88	0.80		

$[6S]_{RBC}$ <> $[6S]_{NaCl}$ $p < 0.047$
$[6R]_{RBC}$ <> $[6R]_{NaCl}$ $P < 0.002$
$[6S]_{RBC}$ <> $[6R]_{RBC}$ $p < 0.169$
$[6S]_{NaCl}$ <> $[6R]_{NaCl}$ $p < 0.206$

RBCs ($p < 0.169$) as well as in sodium chloride solution ($p < 0.905$). A high statistically significant difference, however, could be calculated between $[6S]_{RBCs}$ and $[6S]_{NaCl}$ ($p < 0.047$) and between $[6R]_{RBCs}$ and $[6R]_{NaCl}$ ($p < 0.002$). These differences give evidence that the RBCs play a major role for the bioconversion of CHO-THFA under *in vitro* conditions.

Comparing the areas under the concentration time curves, the coefficient of AUC_{RBC}/AUC_{NaCl} is very similar for [6*S*]- and [6*R*]-CHO-THFA (0.88 to 0.80, respectively) (Table III).

This process seems to be of a slow reaction, because in the first two hours after start of the experiments no difference could be observed between RBCs and sodium chloride solution.

The ratio of the two isomers is influenced by their degradation in the test solutions, but absolutely no indices could be found for any conversion of [6*S*] to [6*R*] or *vice versa,* so the administration of the [6*S*]-isomer will not lead to any loss of action caused by racemization.

The observed binding rates are in accordance with our results obtained from a preclinical trial comparing the binding rate of racemic CHO-THFA to RBCs after i.v. and i.a. administration [9]. In this study we found out that [6*RS*]-CHO-THFA is bound to RBCs in the concentration range from 2.7 to 5.2 µg/ml (after i.v. bolus) at 12.0 to 27.8% (k_{RBC} = 0.14 to 0.39) and from 2.1 to 4.3 µg/ml (after i.a. bolus) at 12.3 to 45.7% (k_{RBC} = 0.14 to 0.84).

The importance of the RBCs for the folate pool is documented by the endogenous parent compound folic acid, which is localized in the RBCs to more than 90% [19].

The present results show that RBCs obviously have influence on the blood plasma levels of free and unbound CHO-THFA with regard to stereoselective binding and bioconversion. The higher binding rate of the [6*S*]-form as well as its prolonged degradation half-life under *in vitro* conditions agree with the difference between [6*S*]- and [6*R*]-CHO-THFA which had been observed in pharmacokinetic studies. So the RBCs certainly act as a subcompartment of the blood for CHO-THFA with regard to drug distribution and metabolism of this class of compounds.

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