Biochemical Pharmacology, Vol. 44, No. 9, pp. 1898–1901, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

Differential stereospecificities and affinities of folate receptor isoforms for folate compounds and antifolates

(*Received* 17 *March* 1992; *accepted* 16 *July* 1992)

Abstract—Two membrane folate receptor (MFR) isoforms are present in human tissues i.e. MFR-1 (e.g. placenta) and MFR-2 (e.g. placenta, KB cells, CaCo-2 cells). MFR-1 was expressed in COS-1 cells and the resulting protein had the same polypeptide molecular weight as the native protein. The affinities of (6*S*) and (6*R*) diastereoisomers of N^5-methyltetrahydrofolate, N^5-formyltetrahydrofolate, and 5,10-dideazatetrahydrofolate as well as folic acid and methotrexate to MFR-1, MFR-2 and placental MFR (MFR-1 plus MFR-2) were determined in terms of the K_i values for their competitive inhibition of the binding of [^{3}H]folic acid to these proteins. The results indicated a striking difference in the stereospecificity of MFR-1 and MFR-2 for reduced folate coenzymes; MFR-2 preferentially bound to the physiological (6*S*) diastereoisomers and MFR-1 bound preferentially to the unphysiological (6*R*) diastereoisomers, while dideazatetrahydrofolate did not show significant stereospecificity for MFR-1. Furthermore, MFR-2 displayed significantly (2- to 100-fold) greater affinities for all the compounds tested compared to MFR-1. Purified placental MFR, a natural source of MFR-1 which contains variable amounts of MFR-2, showed intermediate K_i values for the compounds tested compared with MFR-1 and MFR-2 and stereospecificities similar to MFR-1. These observations demonstrate striking differences in the ligand binding sites of MFR-1 and MFR-2 which could potentially be exploited in the design of MFR isoform specific antifolates.

The classical one-carbon reduced folate carrier is an anion exchange protein present in the plasma membranes of a wide variety of cells. This protein is believed to be involved in the transport of the chemotherapeutic antifolate drug methotrexate (MTX)*, the physiological circulating reduced folate coenzyme, (6*S*) N^5-methyltetrahydrofolate (5-CH_3H_4folate) and N^5-formyltetrahydrofolate (5-$CHOH_4$folate) (leucovorin) with K_t values in the range of 1–5 μM [reviewed in Ref. 1]. The membrane carrier has a relatively low affinity for folic acid, an unphysiological form of the vitamin. A second class of proteins, high-affinity folate binding proteins or membrane folate receptors (MFRs), have a high affinity for folic acid ($K_D < 2$ nM) and a relatively low affinity for methotrexate ($K_D > 100$ nM) [1]. These proteins have been shown to bind 5-CH_3H_4folate in the form of a racemic mixture with a relatively high affinity, comparable to that of folic acid [2–4], and can also transport 5-CH_3H_4folate [5–10]. MFRs have been implicated as the transporters of novel antifolate compounds such as 5,10-dideazatetrahydrofolic acid (DDATHF), 2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolic acid (ICI-198,583) and N^{10}-propargyl-5,8-dideazafolic acid (CB3717) whose intracellular targets are enzymes other than dihydrofolate reductase [11–15].

Two MFR isoforms have identified [16]. They are N-glycosylated polypeptides of M_r approximately 28,000 [2–4], and their protein sequences deduced from their cDNA sequences are approximately 70% homologous. These isoforms are designated herein as MFR-1 (placenta) [16] and MFR-2 (placenta, KB cells, CaCo-2 cells) [17–19]. It was of interest to investigate possible differences in the mode of interaction of reduced folate coenzymes and antifolates with the two MFR isoforms since such differences are potentially important in the design of antifolates that are differentially transported by one or the other receptor isoform. Previous studies [2–4] of the interaction of reduced folates with MFRs were carried out with racemic mixtures of these compounds. Furthermore, those studies do not report the relative affinities of folate coenzymes and antifolates to the two MFR isoforms. We therefore undertook a detailed analysis of the binding of folic acid, MTX and the individual diastereoisomers of reduced folates and DDATHF to MFR-1 and MFR-2. In these studies, we used detergent extracts of crude plasma membrane preparations of KB cells (MFR-2) and COS-1 cells in which the cDNA for MFR-1 was transiently expressed as well as purified MFR from placenta (MFR-1 plus MFR-2).

Materials and Methods

Materials. Folic acid and Norit A charcoal were obtained from J. T. Baker (Baker Analyzed), Phillipsburg, NJ, or from the Sigma Chemical Co., St. Louis, MO. MTX was a gift from Dr. J. A. R. Mead, National Cancer Institute, NIH, Bethesda, MD. The (6*S*) and (6*R*) diastereoisomers of 5-CH_3H_4folate and 5-$CHOH_4$folate were gifts from Drs. A. Melera, F. Marazza and F. Giovannini of SAPEC, S. A., Barbengo, Lugano, Switzerland. [^{3}H]Folic acid was obtained from Moravek Biochemicals, Brea, CA. It was stored at −20° and purified prior to use as described [20]. The radiochemical purity of [^{3}H]folic acid was >99% as determined by rechromatography. The (*R*) and (*S*) diastereoisomers of DDATHF were donated by Drs. G. B. Grindey and C. Shih, Lilly Research Laboratories, Indianapolis, IN. *N*-Glycanase was obtained from the Genzyme Co., Cambridge, MA. All other reagents were of the highest purity available.

Cell culture. COS-1 cells (ATCC CRL 1650), a Simian fibroblast cell line transformed by an origin-defective mutant of SV40 and KB cells, were grown in Dulbecco's Modified Eagle's Medium supplemented with fetal bovine serum (10%, v/v), penicillin (100 U/mL) and streptomycin (100 μg/mL).

Transient expression. The cDNA for MFR-1 was placed in the expression vector pCDM8 after cutting either end of the cDNA at approximately 100 bases upstream or downstream from the initiating and terminating codons at *Avr*II and *Not*I sites, respectively. The cDNA ends were then filled in with Klenow DNA polymerase and the

* Abbreviations: MTX, methotrexate; DDATHF, 5,10-dideazatetrahydrofolic acid; CB3717, N^{10}-propargyl-5,8-dideazafolic acid; ICI-198,583, 2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolic acid; 5-CH_3H_4folate, N^5-methyltetrahydrofolate; 5-$CHOH_4$folate, N^5-formyltetrahydrofolate; and MFR, membrane folate receptor.

resulting 1 kb fragment was inserted in the polylinker region of pCDM8. The vector containing the MFR-1 cDNA in the correct orientation was amplified in *Escherichia coli* MC1061/P3. The resulting plasmid was used to transfect COS-1 cells using lipofectin (Bethesda Research Laboratories) according to the protocol of the manufacturer. COS-1 cells were harvested 48 hr after transfection. Untransfected COS-1 cells were used as the negative control in all experiments.

Preparation of crude plasma membrane extracts. Cells from confluent cultures were washed with 10 mM sodium acetate, pH 3.5/150 mM NaCl (acid buffer) to remove endogenously bound folates from the receptors, followed by washing with 10 mM sodium phosphate buffer, pH 7.5/150 mM NaCl (PBS). The cells were then suspended in 1 mM $NaHCO_3$, 2 mM $CaCl_2$, 5 mM $MgCl_2$ and 1 mM phenylmethanesulfonyl fluoride, pH 7.7 (lysis buffer) at 4°. The cells were allowed to swell for 30 min at 4°, and then were homogenized (50 strokes) in a glass dounce homogenizer. The homogenate was centrifuged for 5 min at 2800 *g* to sediment nuclei and residual cells. The resulting supernatant was centrifuged for 65 min at 40,000 *g* to sediment the membranes. The membranes were resuspended in acid buffer, centrifuged and then washed again with PBS. The resulting crude membrane preparation was dissolved in PBS containing 1% Triton X-100 (assay buffer) by vortexing the mixture thoroughly and centrifuging at 10,000 *g* for 5 min to sediment insoluble material.

Placental MFR. MFR from placenta was purified essentially by affinity chromatography on folic acid-Sepharose as previously described [16].

[^{3}H]*Folic acid binding assay and* K_i *determination.* The [^{3}H]folic acid binding assay was performed essentially as described earlier [16]. An appropriate amount of the sample was incubated with 2.6 pmol [^{3}H]folic acid (sp. act. 28 Ci/mmol) in 0.5 mL of 10 mM sodium phosphate buffer, pH 7.5, 150 mM NaCl, 1% Triton X-100 (assay buffer). After 30 min at 4° unbound [^{3}H]folic acid was removed by adding 0.5 mL of an ice-cold suspension of Norit A charcoal (80 mg/mL in assay buffer). After a further 5-min incubation on ice, the charcoal was sedimented by centrifugation at 14,000 *g* at 4° for 10 min. The supernatant was analyzed for radioactivity using ScintiVerse II liquid scintillation fluid (Fisher Scientific) and a Beckman LS3801 Counter.

For K_i determinations, MFRs were assayed as above in the presence of a range of concentrations of inhibitor and different fixed concentrations of [^{3}H]folic acid. Kinetic analysis of the resulting data was used to calculate K_i values for various inhibitors [21]. In the inhibition assays, possible competition for binding to the charcoal between the inhibitor and [^{3}H]folic acid was routinely monitored by carrying out "blank" assays in the absence of receptor and comparing the values with and without inhibitor for each inhibitor concentration.

Deglycosylation and Western blotting. Appropriate amounts of crude plasma membrane extracts were incubated with 0.3 U of *N*-glycanase (Genzyme) according to the protocol of the manufacturer. Treated and untreated samples were extracted three times with an equal volume of cold 1-butanol saturated with water, to remove Triton X-100. The samples were dried, electrophoresed by standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose. Prestained molecular weight markers (Bethesda Research Laboratories) were transferred simultaneously to the nitrocellulose blots. The blots were treated initially with 10 mM sodium phosphate buffer, pH 7.5/150 mM NaCl/5% non-fat dry milk/0.1% Tween-20 and probed in the same buffer with affinity purified rabbit antibodies to placental MFR and a secondary antibody (goat-anti-rabbit IgG) coupled to alkaline phosphatase according to standard procedures. MFR bands were visualized using nitroblue-tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as substrates.

Results and Discussion

Expression of MFR-1 in COS-1 cells. Although placenta is a rich source of MFR-1, purified preparations of MFR from placenta used in this study contained comparable amounts of MFR-1 and MFR-2 as determined by immunoblots using MFR isoform specific antipeptide antibodies (unpublished results from this laboratory). Most normal tissues express relatively low levels of MFR since cells only require about 2–10 pmol of folate/mg cellular protein for normal growth [7, 20]. It was therefore necessary to use a mammalian expression system as the source of MFR-1 to compare its binding properties with those of MFR-2. The well-studied KB epidermoid carcinoma cells were used as the source of MFR-2. For the purpose of comparison, purified placental MFR (MFR-1 plus MFR-2) was also included in this study as a natural source of MFR-1.

When the cDNA for MFR-1 was expressed transiently in COS-1 cells, they expressed approximately 50–200 pmol of [^{3}H]folic acid binding sites in their plasma membranes/mg of membrane protein. The binding of [^{3}H]folic acid by untransfected COS-1 cells was <5% of that observed in the transfected cells. Detergent extracts of deglycosylated crude plasma membrane preparations from transfected COS-1 cells and untransfected controls were probed with antibodies to placental MFR on Western blots. A band corresponding to MFR-1 was observed for the protein from transfected cells (Fig. 1, lane B) but not in the untransfected control (Fig. 1, lane C). This band migrated similarly to the 28,000 Da protein that resulted from deglycosylation of purified placental MFR (Fig. 1, lane A). These results demonstrate that the MFR-1 polypeptide expressed in COS-1 cells has the same molecular weight as that of the natural protein in placenta. The protein samples used in Fig. 1 were first deglycosylated with *N*-glycanase, an enzyme that specifically cleaves carbohydrate moieties attached to asparagine residues. The nature and amount of N-linked glycosylation conferred on MFR-1 would be expected to vary between COS-1 cells and placental tissues. In fact, N-glycosylation patterns for a given protein are known to vary among different cell types of the same species. However, the affinity of MFR for [^{3}H]folic acid does not appear to be altered upon deglycosylation with *N*-glycanase [16]. It appears that core glycosylation of at least one of the asparagine residues in MFR is needed for the initial folding of the nascent MFR polypeptide, but that the mature protein does not require glycosylation for binding to folate [22]. It is thus assumed in this study that MFR-1 expressed in COS-1 cells has ligand binding characteristics that are similar to the protein expressed by various human tissues.

Interaction of MFR-1 and MFR-2 with folate coenzymes and antifolates. The affinities of folic acid, methotrexate and the diastereoisomers of 5-CH_3H_4folate, 5-$CHOH_4$folate and DDATHF were measured in terms of the K_i values for their inhibition of the binding of [^{3}H]folic acid to MFR-1 and MFR-2 (Table 1). Since, from kinetic analysis, the compounds tested in Table 1 were found to be fully competitive inhibitors of [^{3}H]folic acid binding, the K_i values would also represent the corresponding K_D values for these compounds.

MFRs by definition display a high affinity for folic acid, the unphysiological form of the coenzyme, and a relatively low affinity for the classical antifolate drug, MTX. Accordingly in Table 1, MFRs expressed in COS-1 cells, KB cells and placental MFR showed K_i values of 0.35 to 1.5×10^{-9} M for folic acid and 114 to 1900×10^{-9} M for MTX. To discern possible subtle differences between MFR-1 and MFR-2 in their mode of binding folate compounds, we tested the binding of the (6*S*) and (6*R*) diastereoisomers

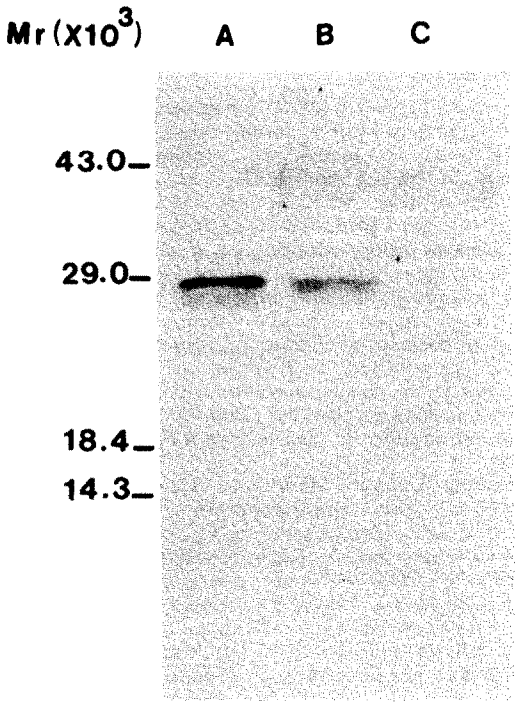

Fig. 1. Western blot of deglycosylated MFRs. Lane A, purified placental MFR; lane B, membrane proteins from COS-1 cells transfected with MFR-1 cDNA; lane C, membrane proteins from untransfected COS-1 cells. Deglycosylation was performed using *N*-glycanase. The blot was probed using an affinity purified rabbit antibody to placental MFR followed by alkaline phosphatase conjugated goat-anti-rabbit IgG, as described under Materials and Methods.

of 5-CH_3H_4folate and 5-$CHOH_4$folate to these proteins. As indicated in Table 1, MFR-2 from KB cells bound the physiological (6*S*) diastereoisomers of 5-CH_3H_4folate and 5-$CHOH_4$folate four times and two times more tightly, respectively, than the corresponding (6*R*) diastereoisomers. On the other hand, MFR-1 expressed in COS-1 cells bound the non-physiological (6*R*) diastereoisomers of 5-CH_3H_4folate and 5-$CHOH_4$folate approximately seven times and twelve times more tightly, respectively, than the corresponding (6*S*) diastereoisomers. The apparent K_i values for placental MFR (MFR-1 plus MFR-2) showed a stereospecificity similar to MFR-1 expressed in COS-1 cells. This could be due to the large differences in stereospecificities and absolute affinities of diastereoisomers of 5-CH_3H_4folate and 5-$CHOH_4$folate between MFR-1 and MFR-2. The results obtained with placental MFR, a natural source of MFR-1, confirm the observations made with MFR-1 expressed by transfected COS-1 cells.

As shown in Table 1, MFR-1 and MFR-2 demonstrated not only different stereospecificities for reduced folate coenzymes but also significantly different affinities for the physiological (6*S*) diastereoisomer of 5-CH_3H_4folate and for the (6*S*) and (6*R*) diastereoisomers of 5-$CHOH_4$folate, i.e. approximately 50-, 100- and 4-fold lower affinities, respectively, for MFR-1 relative to MFR-2. The possible significance of this difference in folate binding in physiological folate uptake is presently unclear. Folic acid, DDATHF and MTX also bound more tightly to MFR-2 than to MFR-1. It is assumed in this study that MFR-1 expressed in COS-1 cells has ligand binding characteristics that are similar to the protein expressed in various human tissues. Accordingly, placental MFR (MFR-1 plus MFR-2) generally showed intermediate K_i values for the compounds tested compared with the KB and the COS-1 cell proteins. These observations point to striking structural differences in the binding sites of MFR-1 and MFR-2.

DDATHF, a novel chemotherapeutic antifolate compound that is currently in clinical trials, bound to MFRs nearly as tightly as folic acid (Table 1). This is consistent with the observation that MFRs offer the preferred route of cellular uptake of DDATHF [11–15] and that low doses of folic acid can reverse the toxicity of DDATHF [23]. In Table 1 the (6*S*) and (6*R*) diastereoisomers of DDATHF bound with comparable affinities to MFRs. It is possible that any stereospecific difference in the mode of interaction of DDATHF with MFRs is masked by the overall high affinity of binding of this molecule to MFR. Thus, it may be possible to observe stereospecific differences in the binding of reduced folate analogs that have a relatively lower affinity for MFRs. On the other hand, it may be possible to exploit differences in the binding sites of MFR-1 and MFR-2 to design MFR isoform specific antifolates. Such antifolates are potentially important in the treatment of certain types of cancers while reducing toxic side-effects due to their uptake by non-target tissues. In fact, recent

Table 1. Inhibition of the binding of [^{3}H]folic acid to MFRs

	K_i ($\times 10^{-9}$ M)*			
	MFR-1 (COS-1 cells)	MFR-2 (KB cells)	Placental MFR	MFR-1† / MFR-2
Folic acid	1.5	0.35	(0.9)‡	4
5-CH_3H_4folate (6*S*)	55.0	1.0	(31.0)	50
5-CH_3H_4folate (6*R*)	7.5	4.0	(3.5)	2
5-$CHOH_4$folate (6*S*)	800.0	7.0	(180.0)	114
5-$CHOH_4$folate (6*R*)	64.0	15.0	(27.0)	4
Methotrexate	1900.0	113.8	(500.0)	17
DDATHF (6*S*)	4.5	0.43	(2.7)	10
DDATHF (6*R*)	6.2	1.3	(4.3)	5

* SD $\leq$ 0.2.

† Ratio of K_i values for MFR-1 and MFR-2 approximated to the nearest integer.

‡ Values in parentheses indicate apparent K_i values for placental MFR which is a mixture of MFR-1 and MFR-2.

studies from this laboratory on the detailed tissue specificities of MFR-1 and MFR-2 in a wide variety of normal and tumor tissues have clearly demonstrated tissue-specific elevation of either MFR-1 or MFR-2 in various tumors [24].

In conclusion, the two known isoforms of membrane folate receptors showed different stereospecificities for reduced folate coenzymes. The isoform that preferentially bound the (6*R*) diastereoisomers of these compounds also had significantly lower affinities for folic acid, reduced folates and antifolates. Structural differences in the ligand binding sites of these receptors should enable the design of receptor isoform specific antifolates.

Acknowledgements—This work was supported primarily by American Cancer Society Grant BE-4 (to M. R.), and in part by NIH Grant CA41461 (to J. H. F.) and NIH Grant CA48091 (to L. E. G.). We thank Lorrie Newman for typing this manuscript. Technical assistance was provided by Douglas Wilhelm.

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