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Ana M. Palacios

Georgia Southern University, Jiann-Ping Hsu College of Public Health, apalacios@georgiasouthern.edu

Rachel A. Feiner

Northeastern University

Robert M. Cabrera

Catalysis Research Laboratory (CaRLa)

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Characterization of folic acid, 5-methyltetrahydrofolate and synthetic folinic acid in the high-affinity folate transporters: impact on pregnancy and development

Ana M. Palacios^{1,2,*}, Rachel A. Feiner³, Robert M. Cabrera^{1,4}

Abstract

Objectives: Folates are B vitamins that are essential for several molecular, cellular, and biological processes, including nucleotide synthesis, methylation, and methionine cycling. The physiological impacts of these processes on health also extend to cell proliferation, folate deficiency anemia, and reduction of the risk of birth defects during pregnancy. The primary objective of this study was to characterize the binding affinities of different folate forms, folic acid (FA), 5-methyltetrahydrofolate (5MTHF), and folinic acid, to the folate receptors α and β , and to the bovine milk folate binding protein. These three dietary forms of folate are found in enriched grains (FA), various fruits and leafy vegetables (folinic acid), and red blood cells (5MTHF).

Methods: The half maximal inhibitory concentration values and binding curves of each of these folates for each receptor were determined.

Results: Our results indicated that FA had the highest affinity for all folate receptors, followed by 5MTHF, and lastly, by folinic acid, examined by several orders of magnitudes.

Conclusion: These data are expected to provide new insights into the therapeutic applications of the different forms of folate in a variety of diseases.

Keywords: 5-methyltetrahydrofolate, folic acid, folinic acid, folate receptor α , folate receptor β , affinity

Introduction

Folates are essential B vitamins involved in one-carbon metabolism^[1]. Folate is the general term used to refer to compounds that contain a pteridine ring connected to a para-aminobenzoic acid and up to 10 glutamic acid residues. Bioactive forms of folate serve as cofactors in purine and thymidine synthesis and are involved in the re-methylation of homocysteine to methionine. Folate assists in the synthesis of S-adenosyl methionine, the universal methyl donor for DNA, proteins, and lipids^[2]. Demands of folates in general, rise 5 to 10 times during pregnancy^[3]. The protective effect of folates in the prevention of neural tube defects (NTDs) has been well documented, but unfortunately, this effect is limited to only 63% to 70% of cases^[4], leaving ~30% of the population without any known option for improving pregnancy

outcomes^[3,5-7]. Folic acid (FA) has been widely used for food fortification and supplementation since the late 1990s predominantly because of the role that FA plays in preventing the development of NTDs. NTDs are common birth defects with distinctive phenotypes involving incomplete neural tube closure leading to abnormalities of the brain and/or spinal cord, the most common examples being anencephaly and spina bifida. Unfortunately, their etiologic basis remains poorly understood^[8,9]. A recent meta-analysis showed that NTD prevalence was significantly less frequent in geographical regions with mandatory FA fortification relative to that in areas with voluntary consumption (33.86 and 48.35 per 100,000 live births, respectively)^[4]. Additionally, FA supplementation during pregnancy has been linked to epigenetic changes during fetal development that may endure throughout the lifespan; however, this mechanism is currently not completely understood and requires further examination^[10].

5-methyltetrahydrofolate (5MTHF) is the naturally occurring reduced form of folate, whereas fortified foods and supplements contain synthetic oxidized folate, termed FA. 5MTHF is the bioactive form of folate that ultimately serves as a methyl donor in the one-carbon metabolism.

Folinic acid (5-formyl tetrahydrofolate) differs from FA in that it occurs naturally in some foods, including dark green leafy vegetables (spinach, broccoli, turnip greens), beans, peanuts, fresh fruits, and others^[11]. Like 5MTHF, s-folinic acid (SFA) is another form of reduced folate and is the formyl derivative of tetrahydrofolate^[11]. The synthetic form was produced in the 1940s^[12] and has been used therapeutically with folate antagonists to reduce their toxicity^[13], as a chemotherapeutic adjuvant with 5-fluorouracil for the treatment of some cancers^[14], in cerebral folate deficiency syndrome, and in autism spectrum disorders^[15]. Fig. 1 shows the structural forms of FA, 5MTHF, and SFA.

¹ Department of Nutritional Sciences, Dell Pediatric Research Institute, University of Texas at Austin, Austin, Texas; ² Department of Health Policy and Community Health, Jiann Ping Hsu College of Public Health, Georgia Southern University, Savannah, Georgia; ³ Department of Behavioral Neuroscience, College of Science, Northeastern University, Boston, Massachusetts; ⁴ Center for Precision Environmental Health, Baylor College of Medicine, Houston, Texas.

* Corresponding to: Ana M. Palacios, 11935 Abercorn Street, Solms 109H, Savannah, GA 31419, USA. E-mail: apalacios@georgiasouthern.edu

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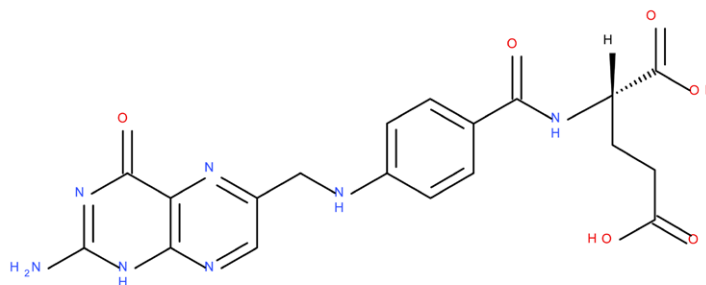
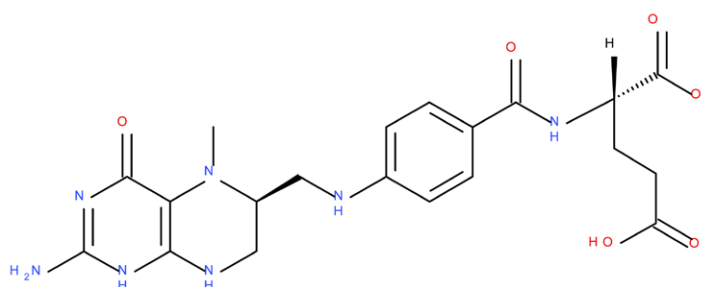
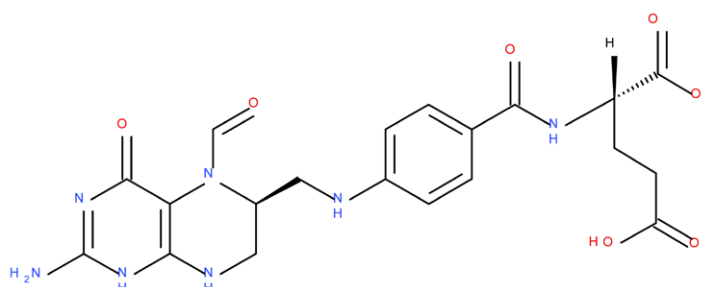
Folic Acid (FA)**5-methyltetrahydrofolate (5MTHF)****s-folinic acid (SFA)**

Fig. 1. Binding curves for folate receptors to folates. Binding was normalized to 100% for each curve and characteristic IC₅₀ values are presented in Tables 1 and 2.

FA is ideal for supplementation owing to the chemical stability of the molecule and ease of its production^[16]. Interestingly, the bioavailability of this component is about 30% to 50% greater than that of naturally occurring folates^[17,18]. Due to their relative instability, storage and cooking of natural sources of folates may decrease their efficacy by 30%^[1].

In humans, three high-affinity folate receptors (FRs) (K_m~1 nM) have been identified. FR α and β are glycosyl-phosphoinositol – membrane anchored proteins that function to import folates into the cellular cytoplasm through receptor mediated endocytosis^[10]. FR α and β are 80% identical and 89% similar, as determined by BLAST sequencing, and both are critically important during embryonic development^[19]. FR α is widely expressed in embryonic tissues, including the yolk sac and placenta^[20,21]. FR β is mainly expressed in the placenta, hematopoietic organs and cells, and its upregulation has also

been identified in the activated macrophages characteristic of some leukemias^[22]. More recently, FR β has become an important therapeutic target in treating autoimmune diseases, like rheumatoid arthritis^[23] and can serve as a measure of inflammatory activation because of its upregulation in activated macrophages^[24].

Bovine milk folate binding protein (bFBP) and FR α share over 80% similarity^[25]. bFBP is highly glycosylated, and at binding saturation, it binds folate 1:1 at the physiologic pH. It has been proposed that one of the roles of bFBP is to divert reserves of folate from plasma to milk to allow for the increased bioavailability of folate during nursing. Additionally, studies have found that bFBP-bound FA was more promptly absorbed in the ileum as compared to unbound FA^[26].

Fbps and FRs determine bioavailability and partition folates into different pathways and tissues. Understanding this

partitioning is essential for the development of effective therapies for cerebral folate deficiency syndrome, autism spectrum disorders, and other conditions that may interfere with folate metabolic pathways, such as FR α auto-antibodies^[27]. A better understanding of these biochemical processes may improve the determination of bioactive folate levels^[28] and contribute to better and more specific therapeutic applications based on the different folate forms.

Materials and methods

bFBP was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), and the recombinant soluble human FR (α or β) was produced using a baculovirus expression system as described and graciously provided by Dr Ratnam^[29]. Briefly, these proteins were cloned with *Apis mellifica* honeybee melittin signal peptide leading sequence using the pENTR Directional TOPO Cloning Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. These vectors were used to produce FR recombinant baculovirus using the BaculoDirect Baculovirus Expression System (Invitrogen). Viral stocks were produced according to the manufacturer's instructions. FRs were expressed in infected Sf9 cells (Invitrogen). For the purification of FR α and FR β from the Sf9 culture medium, the medium was acidified to dissociate folates from FRs. These folates were removed from the media by binding to activated charcoal, and ligand-free FR was captured with methotrexate-agarose (Sigma-Aldrich Co., St. Louis, MO, USA) affinity chromatography.

Gels for western blot and silver staining were resolved by 12% SDS-PAGE alongside Page Ruler pre-stained molecular weight markers (Fermentas, Burlington, Canada). Goat polyclonal human FR antibody C-17 from Santa Cruz Biotechnology, Inc., (Santa Cruz, USA) was used as the primary antibody at 1:1000 for western blot^[30], and a SilverSNAP Stain Kit II (Pierce, Rockford, USA) was used for silver staining^[31].

To measure the different binding affinities of the folates to the receptors, an enzyme-linked immunosorbent assay (ELISA) assay was performed, as previously reported^[25,32]. In short, proteins were printed mechanically onto 96-well polystyrene ELISA plates (Immulon, Thermo Fisher Scientific Inc, Altham, MA, USA) in 1.0 μ L volume with a concentration of 25 ng/ μ L. Plates were incubated at room temperature (20°C) in the dark for 3 hours. Three washes with Tris Buffered Saline pH 8.0 and 0.05% Tween-20 were performed in each well to remove the excess protein.

A competitive standard curve was constructed using serial dilutions of known concentrations of FA (Sigma-Aldrich Co., St. Louis, MO, USA), 5-S-formyltetrahydrofolate, and [6s]-5-methyltetrahydrofolate (Metafolin; Merck Eprova AG, Schaffhausen, Switzerland) and an invariable concentration of FA horse radish peroxidase (FA-HRP) from Vitros Immunodiagnostics, Ortho-Clinical Diagnostics Inc., Raritan, NJ, USA. Plates were washed and incubated with standard curves for 1 hour. Plates were washed again (0.05% Tween-20 followed by Tris buffered saline pH 8.0), and the interaction between the printed FRs, folate curves, and the FA-HRP conjugate was assayed using Super Signal ELISA FEMTO Maximum Sensitivity Substrate (Thermo Fisher Scientific Inc., Altham, MA, USA). Photographs of the plates were taken using Q-View Imager and Software 3.11 (Quansys Biosciences, Logan, UT, USA). Pixel intensities detected were interpolated to the concentrations of each form of folate and plotted using Microsoft Excel. Medians of the pixel intensities were used. All variation coefficients were below 12%. The concentrations of substrates were then logarithmically transformed and plotted

against pixel intensities. The numerical values (IC₅₀) of the half maximal binding of each receptor were calculated. The linear ranges of binding curves were fit to a linear regression function. The R² values of these linear ranges were all greater than 0.975 (data not shown). The half maximal pixel intensity was interpolated into the linear range to calculate the IC₅₀.

Results

Binding affinity curves for folates to high-affinity FRs

The characteristic sigmoidal binding curves of three folate derivatives (FA, 5MTHF, and synthetic SFA) to three high-affinity FRs (FR α , FR β , and bFBP) are presented in Fig. 2. The results of

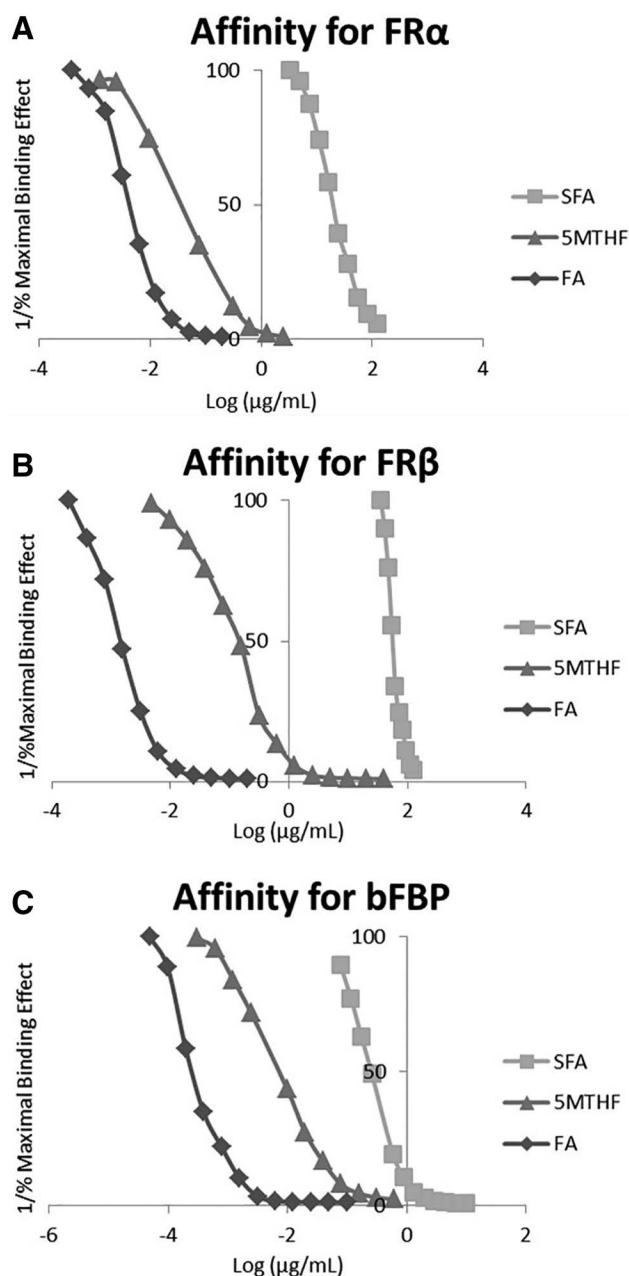


Fig. 2. The three forms of folate are shown. All forms have the same basic pteridine ring, para-amino-benzoic acid, glutamic acid structure. FA has a reduced pteridine ring. 5MTHF has a methyl moiety on an oxidized pteridine ring and SFA has a formyl moiety in the oxidized pteridine ring. 5MTHF: 5-methyltetrahydrofolate; FA: folic acid; SFA: s-folinic acid.

Table 1.
IC 50 values of folates to high-affinity folate receptors.

IC50 (µg/mL)	FR α	FR β	bFBP
Folic acid	0.0015	0.0015	0.0003
5-Methyltetrahydrofolate	0.0500	0.0900	0.0100
Synthetic folic acid	20.9000	12.3593	0.2700

bFBP: bovine milk folate binding protein; FRs: folate receptors.

Table 2.
IC50 values of corrected for total protein concentration in µmol/L.

IC50 µmol/L of printed receptor	FR α	FR β	bFBP
Folic acid	0.0032	0.0032	0.0007
5-Methyltetrahydrofolate	0.1088	0.1959	0.0218
Synthetic folic acid	44.1450	26.1068	0.5703

bFBP: bovine milk folate binding protein; FRs: folate receptors.

a competitive binding ELISA assay are shown for the comprehensive comparison of the ability of each folate to bind to each receptor. Pixel intensity was normalized on a percent scale; thus, each of the curves can be directly compared. The curves show that FA has the highest binding affinity to each receptor and SFA has the lowest binding to each receptor.

The data emphasizes the high binding affinity of FA to all the receptors tested and allows for numerical comparisons of the binding of all the receptors and folates to one another. IC50 values derived from the assays are presented in Tables 1 and 2 for direct comparisons of each derivative and bFBP and FRs.

Discussion

Our results indicate that FA has the highest affinity for all the FRs examined in this study. 5MTHF has the second highest affinity for both FRs α and β , and SFA has the lowest affinity for the FRs by several magnitudes. To maximize the efficacy of therapeutic folate use, the form of folate being utilized should be carefully considered when determining dosages to achieve comparable bioavailable concentrations. Some micronutrient supplements available on the market include FA in combination with other folate forms, such as 5MTHF; however, in some cases, the type of folate and amounts are not well-described on the labels.

FA is used in micronutrient supplements and in the fortification of foodstuffs in more than 88 countries worldwide^[10]. Since 1998, the United States has mandated the fortification of enriched cereal grain products with 140 µg of FA per 100 g of product. Folate fortification has decreased the number of births annually affected by NTDs in North America from 102.56 to 38.70 births per 100,000^[4,33]. This public health success story emphasizes the importance of FA in maternal and reproductive health. Given the demonstrated efficacy, stability, shelf-life, and low cost of FA, it has been selected as the type of folate used for the fortification of foodstuffs in all countries currently practicing folate fortification^[34].

The therapeutic dose range for FA is 400 µg to 8 mg, depending on whether its application is preventive, prophylactic, or whether it is used as a treatment adjuvant^[9]. Levomefolic acid, a calcium salt of 5MTHF, is also commercially available as a folate supplement^[35]. Ocufofolin is another supplement which

contains L-5MTHF, among other micronutrients, which recently has been used in the treatment of diabetic retinopathy and other metabolic processes associated with conjunctival, retinal, and choroidal ischemia^[36]. SFA is used widely as an alternate folate source in individuals undergoing methotrexate and 5-fluorouracil chemotherapies. FA, 5MTHF, and SFA are all ultimately converted into tetrahydrofolate, but through slightly different processes. Naturally occurring folates need to be converted from their polyglutamated forms into a monoglutamic form by hydrolase enzymes in the jejunum prior to being absorbed. FA and 5MTHF are absorbed by enterocytes in the proximal small intestine^[37]. After absorption, FA goes through two reductions with the aid of dihydrofolate reductase (DHFR) to form DHF and then THF, which can be methylated to produce 5MTHF, the folate form found commonly in red blood cells^[38]. Circulating unmetabolized FA has also been documented^[39]. The absorption of FA is primarily facilitated by the proton-coupled folate transporter^[40]. Conversely, folic acid does not require the action of DHFR, making it suitable for concomitant use with DHFR inhibitors^[25]. The alternate pathway utilized by folic acid also makes it a possible candidate for the treatment of pathologies related to polymorphisms of DHFR, as in case some cases of ASD^[27]. Different polymorphisms of methylenetetrahydrofolate reductase (MTHFR) have also been linked to the incidences of various pregnancy outcomes and complications, including pre-eclampsia and preterm birth^[41]. SFA has also been widely used as a therapeutic agent in cerebral folate deficiency syndrome in doses ranging from 1.0 to 5.0 mg/kg/day with promising results^[42].

Our data indicated that because of the lower binding coefficients of SFA to the various FRs, higher doses of SFA may need to be prescribed to deliver a bioequivalent amount of folate. It should also be noted that FA acts as a competitive inhibitor of 5MTHF and SFA^[43]. This should be taken into consideration when medical conditions mandate the preference of one folate form over the others. This study provided insight into cellular folate binding and uptake by FRs and is an important prelude to the expected differences in metabolic impacts of these folates. Specifically, the benefit of FA is expected to be associated with its higher binding affinity to bFBP, FR α , and FR β . It is still unclear if the other tested folates, with lower affinity, would also lead to less downstream signaling compared to those reported between FA and FR α ^[44]. The downstream effect of these interactions are expected to thereby impact stem cell and neural crest proliferation and development^[45]. We previously demonstrated the prevention of pregnancy loss and NTDs achieved by FA and other folates in the *FOLR1* knockout of mice^[19]. These animals present with a range of malformations, including NTDs, craniofacial defects, eye defects, and abdominal wall defects. All of the folates tested in this study are capable of rescuing *FOLR1* knockouts, and curiously, the large differences in *FOLR1* affinity does not translate to observable differences in the doses or concentrations of folate required to rescue these defects. Our interpretation of these differences in molecular interactions and biological impacts is that the other folate uptake pathways, for example, reduced folate carrier and proton-coupled folate transporter, and metabolic pathways appear sufficient to ultimately produce comparable biological impacts regarding reducing birth defect risk in this animal model despite differences in binding affinities. This supports the assurance that FA and reduced folate are all expected to provide reduced risk of folate-related birth defects. In the presence of other genetic interactions, such as *DHFR*

mutations, there are reported differences between the biological impacts of folates^[46,47]. How this may translate to more common variations in folate metabolism enzymes will require either humanized animal or cellular models carrying these genetics variations or head-to-head folate comparisons in human populations, averaged and sub-analyzed based on more common variations. For example, *MTHFR* variations are associated with differences in serum folate and homocysteine, but both 5MTHF and FA improve this outcome in women challenged with infertility^[48]. While hints at differences can be found in the literature, such as increased fertility with 5MTHF and vitamin B12 compared to that with FA alone in women undergoing assisted reproduction^[49], additional studies are needed to obtain definitive conclusion.

The data presented here allow for high throughput and inexpensive characterization of the binding of folates and their derivatives to various FRs. This assay and the standard binding affinities reported set the stage for straightforward comparisons of modified derivatives, drugs, or other receptors. Future research should focus on identifying the affinities of different folates to the reduced folate carrier and the proton-coupled folate transporter.

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None.

Author contributions

Conceptualization, methodology, analysis, investigation, and original draft preparation: A.P. Original draft preparation, writing, reviewing and editing: R.F. Conceptualization, analysis, supervision, writing, reviewing and editing: R.C.

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Conflict of interest

R.C. was a member of TeratOmic Consulting LLC. This organization no longer exists, having been recently dissolved. The organization provided expert witness testimony in birth defect cases. The other authors declare no conflict of interest.

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