IN VITRO DEVELOPMENT OF FOLATE TRANSPORT AND ENDOCYTOSIS BY PCFT, RFC, and FRa, IN A TRANSWELL SYSTEM

Xuexiang "John" Zhang*, Yewei Du, Mark S. Warren, and Steven W. Louie

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Abstract

Scientists continue to explore novel approaches to drug discovery and development for cancer therapeutics. Frequently transporters are exploited to target drug delivery to cancer cells. Folic acid (Vitamin B9) transporters remain a viable transporter candidate to explore anti-cancer drugs. Folic acid is needed for *de novo* synthesis of purines and thymidine for DNA synthesis and repair. Folate or its reduced form is transported by reduced folate carrier (RFC), proton-coupled folate transporter (PCFT), and folate receptor (FR) -a,β,γ. FR-a,β isoforms are membrane bound. FRa on non-cancerous cells is confined to cells crucial for embryonic development, the choroid plexus, and kidneys. However, due to the ubiquitous expression of RFC and PCFT in cancers, folate analogs (antifolates) have been developed for treatment: methotrexate, pemetrexed, pralatrexate are transported into tumor cells by RFC and PCFT. In addition, FRa is expressed 100-300x higher in carcinomas than healthy cells, making this an ideal target to exploit novel drug therapies. FRa is overexpressed in epithelia carcinomas and can be used as tumor-targeted drug delivery system for small molecule-drug conjugates. In fact, many new, folic acid conjugated cytotoxics can be endocytosed, released in endosome, diffused out to intracellular targets. Here, we describe development of *in vitro* folate transporter and receptor assays to potentially support drug targeting. We transiently-transfected PCFT, RFC, or FRa to MDCK cells in 96-well transwells and tested folate and anti-folate probe transport. PCFT was expressed mainly on the apical side and demonstrated both methotrexate and folic acid transport, preferentially at acidic pH. PCFT folate transport showed a 31.7x FOA above mock controls. Bromosulphthalein (BSP) inhibited PCFT folic acid transport with an estimated IC_{50} value of 78.1 μM. No folic acid transport was observed by RFC. However, RFC transported methotrexate from the basolateral side and was inhibitable by BSP and pemetrexed with an IC_{50} value for pemetrexed at 157 μM. We demonstrated FRa receptor-mediated endocytosis of folic acid from both the apical and basolateral sides, resulting in an intracellular folic acid concentration 10.7x above mock controls. Both pemetrexed and BSP inhibited folic acid endocytosis with IC_{50} values of 17.5 μM and 1634 μM, respectively. Taken together, we have developed viable *in vitro* tools to support folate transporter drug discovery as well as the potential to develop other receptor-mediated endocytosis/transcytosis such as asialoglycoprotein receptor (ASGPR), transferrin receptor (TfR), low density lipoprotein receptor (LDLR), or potentially any other membrane bound receptor.

Introduction

Folate, aka vitamin B9, including the dietary folate and synthetic folic acid, plays an important role in numerous vital cellular processes. Through its major circulating active form 5-methylenetetrahydrofolate (5-MTHF), folate is used as a carrier for one carbon fragments in DNA/RNA synthesis, repair and methylation, as well as biosynthesis of the essential amino acid methionine. Folate is especially of importance during rapid cell proliferation. Therefore, a number of folate derivatives known as antifolates, have been developed as anticancer drugs. Methotrexate has been widely used by oral administration in cancer chemotherapy and non-oncological rheumatoid arthritis and psoriasis. Pemetrexed injectable under the trade name of Alimta® is a novel antifolate targeting multiple folate metabolic enzymes.

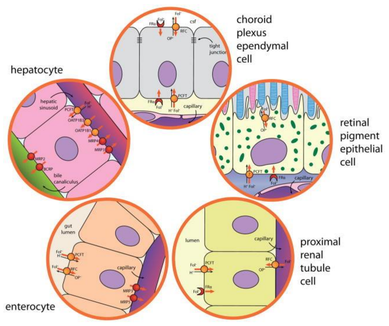


Fig 1. The expression pattern of folate transporters / receptors in epithelial cells (Ref 2).

Folate is a hydrophilic nutrient and an anion at physiological pH, thus its permeability across epithelial cell membrane is low. Its absorption relies on transporters in both the small intestine and colon. Proton-coupled folate transporter (PCFT, SLC46A1) is mainly localized in the upper (acidic) part of the intestine including the duodenum and proximal jejunum. As a proton-folate symporter that couples the uptake of folate to the flow of protons down an electrochemical gradient, the activity of PCFT is higher in low pH. The reduced folate carrier (RFC, SLC19A1) is expressed ubiquitously in mammalian tissues, including intestinal and colonic epithelia. However, it functions optimally at around neutral pH and favors reduced folates, as its name stands for. PCFT seems to be the primary transporter responsible for folate intestinal absorption.

Distribution of folate into the tissues, including the placenta and brain, involves folate receptors in addition to PCFT and RFC. Four isoforms of high-affinity folate binding protein have been reported in humans. Among them, folate receptors FRa and FRβ bind folate and accumulate folate in the intracellular space via endocytosis. FRa is normally expressed in secretory epithelial cells, and it, but not FRβ, is overexpressed on the basolateral surface of tumor cells.

Some ABC transporters in the group of MRPs and SLC transporters in the families of SLC21 and 22 have been reported to be involved in folate absorption and excretion. However, the mechanisms and their contribution remains largely unclear compared to PCFT, RFC and FR.

In this study, we aimed to develop PCFT, RFC uptake assays and FRa endocytosis assay in MDCK-II cells by using transient transfection. The validated *in vitro* assays are suitable for assessing the potencies and characterizing the mechanism of action of antifolates as substrates or inhibitors of folate transporter-mediate transport and receptor mediated endocytosis.

Methods

MDCK-II cells were maintained in DMEM with low glucose and 10% FBS. Cells were seeded in Millipore 96-well insert plates (PCF-0.4 μm) and then transfected using BioIVT proprietary OPTI-EXPRESSION™ technology, an *in situ* transfection allowing consistent and effective transfection of polarized cell monolayers. Cells were either transfected with plasmids encoding PCFT, RFC, or FRa for uptake / endocytosis assays. A plasmid encoding GFP was used as a control. The cells were used in assays 48 hours after transfection to allow sufficient expression and polarization of the transporter of interest in the right side of the cells. The cells were pre-incubated with HBSS for 30 min. Transport was initiated by adding radiolabeled probe substrate in the apical or basal or both chamber(s) of the transwell plate. Following incubation, the cells were washed and solubilized using a 1:1 acetonitrile : water mixture to measure the intracellular accumulation of the radiolabeled substrate by radiometry.

Results

PCFT transport of folic acid

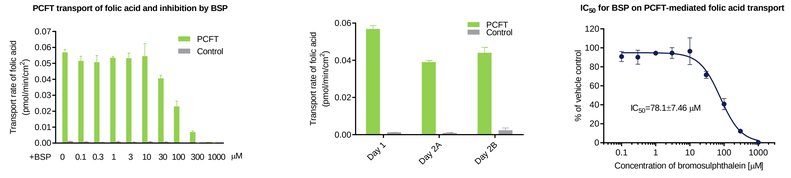


Fig 2. PCFT transport of folic acid. PCFT was expressed on the apical side of MDCK cells. By using 10 nM folic acid at pH 5.5, a robust 31.7X assay window (18.3% CV) was observed in PCFT-transfected cells compared control cells. The transport was inhibited by bromosulphthalein (BSP) with an IC_{50} of 78.1 μM.

PCFT transport of methotrexate

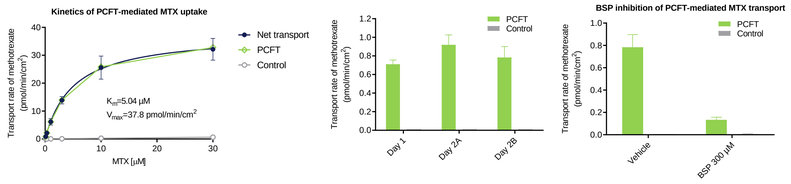


Fig 3. PCFT transport of methotrexate. By using 0.1 μM MTX at pH 5.5, a robust 122X assay window (15.4% CV) was observed in PCFT-transfected cells compared control cells. The transport was inhibited by BSP.

RFC transport of methotrexate

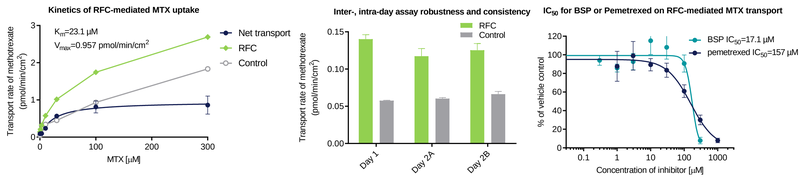
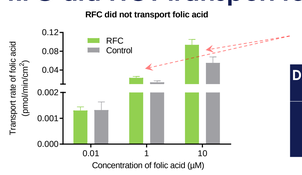


Fig 4. RFC transport of methotrexate. RFC was expressed on the basolateral side of MDCK cells. At pH7.4, high background transport of MTX was observed in control cells, suggesting there might be expression of canine Rfc or other MTX-transporting transporters in MDCK cells. By using 0.5 μM MTX at pH7.4, a 2.08X assay window (21.7% CV) was obtained, and the transport was inhibited by BSP or pemetrexed.

RFC did NOT transport folic acid



The difference of RFC *vs.* control seen at 1 and 10 μM were just at the background noise level (see raw CPM data below)

Dosing conc. of folic acid (μM)	Raw CPM value of intracellular [^{3}H]-folic acid					
	RFC cells			Control cells		
0.01	14	15	14	16	15	13
1	27	28	23	17	21	17
10	27	32	37	30	29	30

Fig 5. RFC did not transport folic acid. At three concentrations of folic acid tested of 0.01, 1 and 10 μM, no significant difference was observed in RFC-expressing cells compared to control cells. This is consistent with literature report and matches the nomenclature of RFC as a carrier for reduced folates.

FRa expressed on both sides of MDCK cells

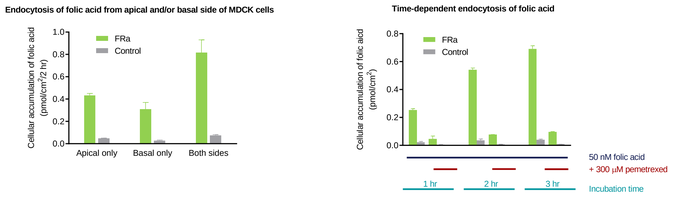


Fig 6. Cellular accumulation of folic acid in FRa cells. Cellular accumulation of folic acid was observed when doing 50 nM folic acid from apical and/or basal side(s), suggesting the expression of FRa on both sides. Endocytosis was linear within 3 hours. Bound but not endocytosed radiolaleled folic acid was removed by extra wash with10 μM unlabeled folic acid for 15 min. Endocytosis of folic acid was inhibited by BSP.

FRa-mediated endocytosis of folic acid

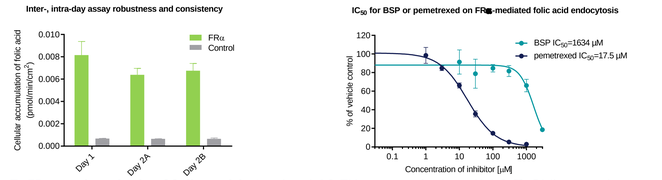


Fig 7. FRa endocytosis of folic acid. A robust 10.7X assay window (16.9 % CV) was observed by using 50 nM folic acid in FRa-transfected cells compared control cells. The cellular accumulation of folic acid was inhibited by BSP and pemetrexed.

Conclusions & Discussion

MDCK-II cells based PCFT and RFC transport assays and FRa endocytosis assay have been successfully developed and validated. BioIVT's proprietary OPTI-EXPRESSION technology and related assay platform are not only applied to conventional transporter assays, but also good for receptor endocytosis, and potentially transcytosis.

PCFT is expressed on the apical side of MDCK-II cells and is optimal at acidic pH (pH5.5) for transport of both folic acid and methotrexate. The assay is robust with 31.7X and 122X assay windows for folic acid and methotrexate, respectively.

RFC is predominantly expressed on the basolateral side of MDCK-II cells and performs better at neutral pH. There might exist abundant endogenous canine Rfc or other methotrexate-transporting transporters, rendering a mere 2.08X assay window for methotrexate transport. However, the assay window is wide enough to generate meaningful IC_{50} inhibition curves for BSP and pemetrexed.

FRa seems equally expressed on the apical and basolateral side of MDCK-II cells. Folic acid endocytosis is linear within 3 hours tested, and cellular accumulation of folic acid is inhibited by BSP and pemetrexed.

Using the validated assays, a number of integrase inhibitors were assessed for potencies on folate pathways, and clinical extrapolation predicted no concerns of decreases in maternal and fetal folate levels (ref 1).

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CONTACT
Xuexiang Zhang | xzhang@bioivt.com | 201-993-9386