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Curr Eye Res. Author manuscript; available in PMC 2013 August 01.

Published in final edited form as:
Curr Eye Res. 2011 May ; 36(5): 404–416. doi:10.3109/02713683.2011.566411.

Functional Characterization of Folate Transport Proteins in Staten's Seruminstitut Rabbit Corneal Epithelial Cell Line

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Abstract

Purpose—The overall objective of this study was to investigate and characterize the expression of folate transport proteins in Staten's Seruminstitut rabbit corneal (SIRC) epithelial cell line.

Methods—[^{3}H]Folic acid uptake was studied with respect to time, pH, temperature, sodium and chloride ion dependency. Inhibition studies were conducted with structural analogs methyltetrahydro folate (MTF) and methotrexate (MTX), vitamins and metabolic inhibitors. [^{3}H]Folic acid uptake was also determined with varying concentrations of cold folic acid. Uptake kinetics was studied in the presence of various modulators of intracellular regulatory pathways; protein kinases A and C (PKA and PKC), protein tyrosine kinase (PTK) and calcium-calmodulin modulators. Reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis were performed to substantiate the expression of folate transport proteins. *Ex vivo* corneal permeability studies were carried out with [^{3}H]Folic acid in presence and absence of 1mM cold folic acid.

Results—Linear increase in [^{3}H]Folic acid uptake was observed over 30min. The process followed saturation kinetics with apparent K_m of 14.2 nM, V_{max} of $1.5*10^{-5}$ micro.moles/min/mg protein and K_d of $2.1*10^{-6}$ min.$^{-1}$ Uptake process was found to be dependent on pH, sodium ions, chloride ions, temperature and energy. Uptake was inhibited in the presence of structural analogs (cold folic acid, MTF and MTX) but structurally unrelated vitamins did not show any effect. Membrane transport inhibitors SITS, DIDS, probenecid and endocytic inhibitor, colchicine, significantly inhibited the [^{3}H]Folic acid uptake indicating the involvement of receptor/transporter mediated process. PKA, PTK and Ca^{2+}/calmodulin pathways significantly regulate the process. RT-PCR and Western blot analysis confirmed the presence of folate receptor-α (FR-alpha) and proton-coupled folate transporter (PCFT). Permeability of [^{3}H]Folic acid across rabbit cornea was$1.48*10^{-05}$ cm/sec, and in the presence of cold folic acid it was $1.08*10^{-05}$ cm/sec.

Conclusions—This work demonstrated the functional and molecular presence of FR-alpha and PCFT in SIRC epithelial cell line. Permeability studies have indicated the existence of folate carrier mediated system across rabbit cornea.

Keywords

folate receptor-alpha; proton coupled folate transporter; reduced folate carrier; cornea; SIRC cells

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Introduction

Folate is a water soluble vitamin B_9 that occurs naturally in food, and folic acid (FA) is the synthetic form of this vitamin. It is found in food supplements and this vitamin plays an essential role in a variety of vital cellular processes. The compound aids in growth, differentiation and homeostasis of mammalian cells. It acts as a coenzyme for synthesis and repair of DNA, RNA, and proteins.[1] FA also plays a vital role in the interconversion of amino acids such as serine and glycine; and biosynthesis of nucleic acids such as adenine, guanine, thymidine and inosine.[2] It plays an important role in the development of visual system, and deficiency of FA results in loss of visual function due to optic neuropathy and nutritional amblyopia.[3]

Several transport systems have been recognized on the cell membranes that play a critical role in internalization of folates. Every system employs a definite set of membrane proteins that binds to their respective substrates with high affinity and specificity.[2] These systems are classified into two types: a) membrane channels or carriers that vectorially drive the molecules, and b) endocytotic vesicles that internalize the molecules. These transporters, due to high efficiency, are often secluded from supplementary molecules in the cell membrane to form domains that are enriched with that transporter species. The transport systems can be distinguished by their preference to a variety of folate compounds as substrates, and more over by differences in temperature and pH dependent uptake. [2,4,5] Three distinct cellular mechanisms for the transport of folate have been identified: folate receptors (FR), reduced folate carrier (RFC), and the newly identified proton-coupled folate transporter (PCFT). Folate receptors are highly specific forms of folate binding proteins, which are fastened to the cell membrane by glycosylphosphatidylinositol (GPI) residues. FR is coded by two specific genes (FR α and FR β) with differential tissue expression.[6] After binding to FR, the folate receptor-complex is internalized into the cell by an endocytotic process. FR contains about 240-260 amino acids and has a molecular mass in the range of ~28-40 kDa, reflecting the extent of glycosylation. The receptor displays much greater affinity for non reduced folates, such as folic acid, over reduced folates like methotrexate (MTX) and methyltetrahydrofolate (MTF). RFC is a 57-65 kDa integral transmembrane protein which shows high affinity for N5-methyltetrahydrofolate (MTF). Reduced folate transporter (RFC) belongs to the SLC19 family of solute carriers *(SLC19A1)*.[1] RFC functions as an anion exchanger operating optimally at pH 7.4. Its activity and folate-concentrating ability are lower at reduced pH.[1] PCFT is a newly described folate transport protein that is encoded by the *SLC46A1* gene. Recently, Goldman's laboratory has discovered the molecular identity of PCFT transporter which is genetically unrelated RFC.[7] It is a proton-coupled, electrogenic transporter and works optimally at low pH. PCFT is reported to have a molecular weight of 50-65 kDa, depending on the extent of glycosylation.[8] It is also known as heme carrier protein 1 (PCFT/HCP1) and has been identified as a transporter that mediates the translocation of folates across cellular membrane.[8]

The purpose of this study was to investigate and characterize the expression of folate transport proteins in Staten's Seruminstitut rabbit corneal (SIRC) epithelial cell line. *In vitro* uptake studies were performed in a rabbit corneal cell line, SIRC. This cell line has been widely considered as a model corneal membrane for evaluation of corneal physiology, immunology, toxicology, and transport. SIRC cell line forms 5-6 layers of epithelium in culture as characterized and reported previously from our laboratory. Thus it serves as a good *in vitro* model for the corneal epithelium.[9] SIRC has not been investigated previously for the presence of folate membrane transporters/ receptors. Herein, we determined kinetic parameters of folic acid, in SIRC cells to identify folate transport proteins on the corneal epithelium. These results were further confirmed by reverse transcriptase polymerase chain

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reaction (RT-PCR) and Western blot studies. Transport studies were also conducted on freshly excised rabbit cornea.[10]

Materials and methods

Materials

[^{3}H]Folic acid (50 Ci/mmol) was purchased from Perkin-Elmer (Boston, MA). Unlabelled folic acid, methyltetrahydrofolate (MTF), methotrexate (MTX), biotin, ascorbic acid, riboflavin, pantothenic acid, sodium azide, ouabain, 2,4-dinitrophenol, protein tyrosine kinase (PTK) modulators (genistin and genistein), protein kinase (PKC and PKA) pathway modulators (bisindolylymaleimide-I, phorbol-12-myristate-13-acetate, forskolin, and 3-isobutyl-1-methylxanthine (IBMX), calcium–calmodulin pathway modulators (calmidazolium,KN-62 and trifluoperazine), probenecid, 4,4′-di-isothiocyanatostilbene-2,2′-disulphonic acid (DIDS), 4′-acetamido-4-isothiocyanostilbene-2,2′-disulfonic acid (SITS), colchicine, choline chloride, Triton X-100, HEPES, d-glucose and all other chemicals were procured from Sigma Chemical Co. (St. Louis, MO). All chemicals were of special reagent grade and used without further purification.

Cell culture

SIRC cells were obtained from ATCC (passages 410). Cells were grown in culture media consisting of minimum essential medium (MEM) supplemented with 15% non-heat-inactivated calf serum, lactalbumin, HEPES, penicillin (100 μg/mL) and streptomycin (100 μg/mL) (Sigma Chemical Co., St. Louis, MO). Medium was replaced on every alternate day. Cells were maintained at 37°C, in a humidified atmosphere of 5% CO_2 and 90% relative humidity. For uptake studies cells were plated at a density of 500000 cells/well on 12-well culture plates (Costar, Corning, NY) and maintained at 37°C.[11]

Uptake studies

SIRC cells were washed three times with 2 mL of Dulbecco's modified phosphate buffer saline (DPBS) at 37°C for 10 min. Folic acid uptake was initiated by the addition of a fixed amount of [^{3}H]-Folic acid (0.5 μCi/mL) at 37°C and cells were incubated for a definite time period. Following incubation, cells were washed thrice with ice-cold stop solution (200 mM KCl and 2 mM HEPES) to terminate the folic acid uptake. Cells were lysed overnight with 1 mL of 0.1% (v/v) Triton X-100 in 0.3 N sodium hydroxide at room temperature. Aliquots (500 μL) from each well were then transferred to scintillation vials containing 5 mL scintillation cocktail (Fisher Scientific, Fair Lawn, NJ). Samples were then analyzed by liquid scintillation counter (model LS-6500, Beckman Instruments, Inc., Fullerton, CA). The amount of protein in the cell lysate was measured by BioRad protein estimation kit (BioRad, Hercules, CA). The rate of uptake was normalized to the protein content of each well.[12-14]

Time dependency

Uptake of [^{3}H]Folic acid was determined at various time points (1, 2, 5, 10, 15, 30 and 45 min) to optimize the time required for carrying out further studies.

Effect of pH and temperature

In order to carry out pH dependent studies, pH of DPBS was adjusted to 4, 5, 6, 7.4, and 8. To determine the effect of temperature on the uptake buffer temperatures were adjusted to 4, 25, and 37°C. Uptake of [^{3}H]Folic acid (10 nM) in SIRC cells was performed under varying pH and temperature conditions.

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Role of ions

Sodium ion dependency was studied by the addition of equimolar quantities of potassium, ammonium and choline chloride to substitute sodium chloride (NaCl) and sodium phosphate monobasic (Na_2HPO_4), in DPBS, pH 5. Effect of chloride ion was also studied by incorporating equimolar quantities of sodium phosphate dibasic (NaH_2PO_4), potassium phosphate (KH_2PO_4), and calcium acetate as substitute for NaCl, potassium chloride (KCl) and calcium chloride ($CaCl_2$), respectively. Uptake was performed in these buffer solutions containing [^{3}H]Folic acid (10 nM).

Effect of energy modulators

To examine the energy dependence, SIRC cells were pre-incubated with 1mM metabolic inhibitors such as ouabain (an inhibitor of Na^+/K^+-ATPase), 2, 4-dinitrophenol (intracellular ATP reducer) and sodium azide (an inhibitor of oxidative phosphorylation) for 1 h. Uptake studies were then initiated as described earlier with buffer solutions containing [^{3}H]Folic acid (10 nM).

Effect of membrane transport inhibitors

To study the effect of anion transport inhibitors on cellular uptake folic acid, cells were first pre-incubated with probenecid, DIDS, SITC at 0.5 mM concentration. To explain the role of folate receptor, cells were incubated with colchicine (100 μM) an endocytotic inhibitor. Uptake studies were then carried out as described earlier with buffer solutions containing [^{3}H]Folic acid (10 nM).

Substrate specificity

In order to understand the structural requirements for interaction with folate carrier, uptake studies were carried out in the presence of various vitamins and its structural analogs. The unlabeled vitamin or structural analog was incubated with respective radiolabelled folic acid (10 nM). Unlabeled vitamins (biotin, pantothenic acid, riboflavin and niacin) were used at a concentration of 10 μM. Unlabeled folic acid and its structural analogs (MTF and MTX) at a concentration of 0.1 and 1.0 μM were added to the incubation mixture.

Concentration dependent study data analysis

Uptake of [^{3}H]Folic acid was carried out in presence of various concentrations of cold folic acid and the data obtained was fitted into a modified Michaelis–Menten equation (Eq. 1). This equation considers the carrier-mediated active uptake process as a non-saturable passive diffusional uptake process:

$$V=\frac{V_{\max}[C]}{K_m+[C]}+K_d[C] \quad \text{Eq.1}$$

V represents the total rate of uptake of folic acid, $V_{\max}$ denotes the maximum uptake rate of the carrier-mediated process, K_m (Michaelis–Menten constant) is the substrate concentration at halfmaximal saturation process, C is the substrate concentration, K_d represents rate constant for the non-saturable (passive) diffusion component and $K_d[C]$ represents the non-saturable (passive) component, whereas the saturable component of total uptake of folic acid is given by ($V_{\max}[C]$)/(K_m + [C]). Data was fitted into Eq.1 with a SCIENTIST® program (Micromath, St. Louis, MO, USA). The kinetic parameters which were calculated with SCIENTIST® were substituted into above equation to determine the involvement of the saturable and non-saturable components. The excellence of the fit was examined by

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evaluating the coefficient of determination (R^2), the standard error of parameter estimates, and by visual inspection of the residuals.

Statistical analysis

All experiments were conducted at least six times and results were expressed as mean ± S.D. Michaelis–Menten parameters such as K_m, K_d and V_{max} are expressed as mean ± S.E. Unpaired Student's *t*-test was used to estimate statistical significance. A difference between mean values was considered significant if $p < 0.05$.

Intracellular regulation

Involvement of intracellular regulatory pathways such as protein kinase C (PKC), protein kinase A (PKA), protein tyrosine kinase (PTK), and Ca^{2+}/calmodulin-mediated pathways in the regulation of [^{3}H]Folic acid uptake into SIRC cells was determined. Cells were first pre-incubated for 30 min separately with PKC pathway activator (phorbol 12 myristate 13-acetate), or with the PKC pathway inhibitor (bisindolylmaleimide I), PTK pathway modulators (genistein and genistin), PKA pathway modulators activators (IBMX and forskolin and specific inhibitor, H-89), calmodulin inhibitors (calmidazolium and tri-fluoperazine) and Ca^{+2}/calmodulin dependent protein kinase II inhibitor (KN-62). Solutions of these modulating agents were prepared in DMSO or pure ethanol (final concentration of the organic solvent was less than 1%, v/v). Cells were preincubated for 1 h with various modulators and uptake was initiated by the addition of [^{3}H]Folic acid (10 nM). An identical amount of drug-dissolving vehicle (DMSO or ethanol) was incorporated in the bathing medium for control studies to determine the effect of these solvents on untreated SIRC cells.

Molecular evidence

Gene expression – qualitative analysis—Total RNA was isolated from SIRC cells using Trizol® reagent (Invitrogen) by a standard protocol. Briefly, cells were lysed with the addition of 800 μL of Trizol® reagent. The lysate was then transferred to Eppendorf tubes. RNA was extracted by the phenol–$CHCl_3$–isopropanol method, purified, and dissolved in 50 μL of RNase–DNase-free water. For single strand cDNA synthesis, 2 μg total RNA was reverse transcribed according to a standard protocol using MMLV Reverse transcriptase (Promega, Madison, WI). The conditions for reverse transcription were denaturation of template RNA for 2 min at 94°C and reverse transcription for 60 min at 40°C. Amplification was performed with 1 μg cDNA and selected primers for the amplification were shown in Table 1. GAPDH served as the internal control. PCR conditions were as follows: denaturation (94°C, 45 s), annealing (55°C, 1 min), and extension (72°C, 45 s) for 35 amplification cycles, followed by a final extension of 72°C for 10 min. The product was separated by 1.5% agarose gel electrophoresis and visualized by chemiluminescence.

Computer analysis—Nucleotide sequence homology matching was carried out with a fundamental local alignment investigating tool (BLAST) via on-line link to the National Center of Biotechnology Information (NCBI).

Western blot analysis for folate transport proteins—Protein was extracted from SIRC cells as follows: Cells were washed thrice with DBPS (pH 7.4), centrifuged at 1000 *g* for 3 min, suspended in protein extraction buffer and incubated on ice for 30 min. The lysate was homogenized for 30 sec followed by centrifugation at 8,000 g for 15 min. Protein samples were subjected to SDS-PAGE and, after transfer to nitrocellulose membranes, were incubated with antibody against FR-α, PCFT, or RFC overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated goat rabbit-anti goat IgG antibody for FR- α and goat-anti rabbit IgG for PCFT and RFC. After washing, with PBST, protein

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expression was visualized with the Super Signal West Pico Chemiluminescence detection system (Thermo Scientific, Rockford, IL). β-actin served as the loading control.

Tissue Preparation—Dutch Belted Pigmented rabbits weighing between 2 and 2.5 kg were obtained from Myrtle's Rabbitry (Thompson Station, TN). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of University of Missouri- Kansas City (UMKC, Kansas City, MO). Rabbits were anesthetized with intramuscular administration of ketamine HCl (35 mg/kg) and xylazine (5 mg/kg). Then the animals were euthanized by an overdose of sodium pentobarbital (100 mg/kg) administered through marginal ear vein under deep anesthesia. Eyes were removed carefully and a small incision was made on the sclera. The cornea was excised by removing lens and iris-ciliary body. The cornea was placed in a petridish and washed with Dulbecco's phosphate buffered saline.

Permeability Studies—The excised cornea was mounted on a Side-by-Side diffusion apparatus for carrying out permeability studies. The corneal side was placed towards the donor chamber in which 3.0 mL of drug solution ([^{3}H]Folic acid, 10 nm) was placed. The receptor chamber was filled with 3.2 mL of DPBS. Permeability experiment was carried out at 34°C (*in vivo* corneal temperature). The receptor chamber was maintained at a higher volume (3.2 mL) so as to maintain hydrostatic pressure sufficient to maintain corneal curvature. For competitive inhibition studies cold folic acid at a concentration of 1mM was added. An aliquot (100 μL) was withdrawn at regular time intervals and replaced with equal volume of fresh buffer. All the experiments were carried under sink conditions. Simultaneously, [^{3}H]-mannitol permeability studies were carried out to observe the integrity of corneal tissues. The samples were transferred into scintillation vials, mixed with 5 mL scintillation cocktail and analyzed for radioactivity with the help of a scintillation counter (Model LS-9000; Beckman Instruments, Inc.). All the permeability studies were carried out in triplicate.

Permeability (P_{app}) of folic acid was calculated using Eq. 2.

$$\text{Permeability } (P_{\text{app}}) = \text{Flux}/C_d \quad \text{Eq. 2}$$

Flux (J) is calculated by dividing the slope obtained by plotting cumulative amount of folic acid permeated (M) through the cornea vs time (t) with cross sectional area of the membrane (A) exposed to the drug. C_d represents the initial folic acid concentration in the donor chamber.

Results

Time dependent uptake

Time-dependent uptake of [^{3}H]Folic acid (10 nM) was carried out in SIRC cells. As shown in Fig. 1, the uptake increased linearly up to 30 min and reached equilibrium at 45 min. Hence all uptake experiments were carried out over 30min unless otherwise mentioned.

pH dependence

Role of hydrogen ions on uptake of [^{3}H]Folic acid was examined by regulating the buffer pH over a range of 4–8. Uptake of folic acid was highest at acidic pH of 4 and 5 suggesting that the uptake process is probably driven by a proton gradient. There was a considerable decrease in the uptake of folic acid at pH 6, 7 and 8 (Fig. 2). The process could be mediated by PCFT transporter which requires proton coupling for the transport of folic acid. Since the

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folic acid uptake was significantly higher at acidic pH all further uptake experiments were carried out at pH 5.

Temperature dependence

The uptake experiments were carried out at three different temperatures (4, 25 and 37°C). As indicated in Fig. 3, the rate of uptake significantly reduced at 4 and 25°C relative to 37°C. This is due to the arrest of cellular energetics at lower temperatures. At 4 °C the uptake of folic acid is very low. This may be due to the arrest of receptor mediated endocytosis. This study clearly indicates that folic acid uptake is temperature dependent and the uptake is optimum at a physiological temperature, 37°C.

Role of ions

In order to delineate ion dependency on folic acid uptake mechanism, Na^+ ions in the medium were replaced with equimolar quantity of K^+, NH_4^+ and choline chloride; while Cl^- ions were replaced with salts of alternative organic and inorganic monovalent anions (phosphate and acetate). There was a significant difference in the uptake of folic acid in the absence of Na^+ and Cl^- ions. Figure 4 clearly illustrates Na^+ and Cl^- ion dependency of the uptake process.

Role of energy inhibitors

The effect of metabolic inhibitors on the uptake of [^{3}H]Folic acid was examined. A Na^+/K^+-ATPase inhibitor (ouabain), intracellular ATP reducer (2, 4-dinitrophenol; DNP) and oxidative phosphorylation inhibitor (sodium azide) were used as metabolic inhibitors. Fig.5 shows that the uptake process was significantly inhibited in the presence of all energy inhibitors indicating that the process is highly energy dependent.

Role of membrane transport inhibitors

Role of membrane transporter inhibitors was investigated by incubating the cells with probenecid, DIDS and SITC and endocytosis process inhibitor colchicine at 0.5 mM concentration. As indicated in Fig. 6, there was a significant lowering in folate uptake in the presence of membrane transport inhibitors indicating the involvement of anion exchanger. Moreover, significant inhibition in uptake of folic acid in presence of 1 μM colchicine suggests the involvement of receptor mediated endocytosis. This result suggests the existence of both folate transporter and receptor on SIRC cell line.

Substrate specificity

Substrate specificity of the saturable uptake process was investigated in presence of various structural analogs on folic acid. The effect of these compounds on the uptake of [^{3}H]Folic acid was significant. Uptake rate of folic acid is shown in Fig.7 and was found to be 26.96±0.12, 4.88±0.02, 3.36±0.25,9.22±0.26, 5.02±1.54, 12.46±0.15, 9.20±0.96, 26.39±0.9, 25.52±3.05, 24.90±3.84, 27.97±1.53 and 25.73±3.84 fmol/(min mg) protein for control and in the presence of folic acid (0.1 μM), folic acid (1 μM), MTF (0.1μM), MTF (1 μM), MTX (0.1 μM), MTX (1 μM), biotin (10 μM), pantothenic acid (10 μM), riboflavin (10 μM) and niacin (10 μM), respectively. As shown in Fig.7, [^{3}H]Folic acid uptake was significantly reduced in the presence of 0.1 and 1μM of unlabeled folic acid. Significant inhibition was also observed with MTF and MTX at a concentration of 0.1 and 1 μM. Unlabeled vitamins (biotin, pantothenic acid, riboflavin and niacin) did not show any effect on the uptake process.

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Concentration dependency

The existence of a carrier-mediated system for folic acid in the SIRC cell line was determined by evaluating the uptake kinetics of folic acid in the presence of unlabeled folic acid. Total [^{3}H]Folic acid uptake was analyzed and the data illustrates that the uptake mechanism consists of two pathways: a saturable pathway (carrier mediated) at lower concentrations and an apparently a non-saturable (passive) pathway that dominates over carrier mediated process at higher concentrations above 0.1 μM for folate (Fig. 8). Saturable as well as non-saturable components were determined by substituting the values of the kinetic constants into Michaelis–Menten equation. Uptake process by the saturable components was determined by subtracting the diffusional component from the total uptake at each concentration. After fitting the data to modified Michaelis–Menten equation, an uptake process with apparent K_m of 14.2 nM, V_{max} of $1.5*10^{-5}$ micromoles/min/mg protein and K_d of $2.1*10^{-6}$ min^{-1} were obtained.

Role of intracellular regulatory pathways

The role of Ca^{+2}/calmodulin-mediated pathways in the regulation of [^{3}H]Folic acid uptake was examined in SIRC cells by pre-treating them with calmodulin inhibitors (calmidazolium and trifluperazine) and with Ca^{+2}/calmodulin dependent protein kinase II inhibitor (KN-62). These compounds significantly ($p < 0.05$) reduced the folic acid uptake in a concentration dependent manner as shown in Fig.9. Role of PKA-mediated pathway in the regulation of folic acid uptake was also studied by pre-treating SIRC cells for 1 h with compounds that are known to increase intracellular cAMP levels (3-isobutyl-1-methylxanthine and forskolin) thus activating PKA. This study clearly indicated that 3-isobutyl-1-methylxanthine and forskolin significantly ($p < 0.05$) inhibited uptake of folic acid in a concentration dependent manner. The effect of specific PKA inhibitor H-89 on the folic acid uptake was also examined. Forskolin and IBMX induced PKA activity was abolished by H-89 as shown in Fig. 10. Reduction in folic acid uptake by cAMP modulators suggested the involvement of cAMP-dependent protein kinase A (PKA) in the regulation of folic acid transport. In another study, we examined the involvement of PTK pathway in the regulation of folic acid uptake in SIRC cells which were pretreated for 1 h with the PTK inhibitor, genistein. Genistin is considered as a negative control for this inhibitor. There was significant difference in the uptake of [^{3}H]Folic acid in the presence of genistein (10– 100 μM) as shown in Fig. 11. These results suggest that Ca^{+2}/calmodulin, PKA and PTK pathways play a significant role in the translocation of folic acid into SIRC cells.

Molecular evidence: RT-PCR analysis

Expression of the folate carrier systems in SIRC cells at the mRNA level was determined by RT-PCR analysis (Fig. 12). Gel electrophoresis had shown two major bands (547bp, 407bp) corresponding to the amplified folate receptor alpha (lane 3, 4). No product was observed for reduced folate carrier (lane-5). Similarly two major bands (625bp, 624bp) were obtained for proton coupled folate transporter (PCFT: lane 6, 7). BLAST analysis showed that the primers used in this study can result in a PCR product size as specified.

Western blot analysis

Western blot analysis (Fig. 13) indicated the expression of folate receptor-alpha protein at 40 kDa molecular weight and a clear distinct band was observed for PCFT at 50 kDa. Hence this result confirms the existence of FR-α and PCFT proteins in SIRC cells.

Tranport across rabbit cornea

Transport of [^{3}H]Folic acid across rabbit cornea in the presence and absence of 1mM concentration of cold folic acid is shown in Fig. 14. Permeability of [^{3}H]Folic acid across

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rabbit cornea was found to be $1.48*10^{-05}$ cm/sec (Table 2), and in the presence of cold folic acid it was $1.08*10^{-05}$ cm/sec (Table 2). This study further corroborates the existence of folate carrier mediated system on the rabbit cornea.

Discussion

The aim of this study was to identify a folate carrier mediated system in the Staten's Seruminstitut rabbit corneal (SIRC) epithelial cell line and to evaluate it as an *in vitro* screening tool for regulating the mechanism and intracellular regulation of folic acid uptake. Previous reports suggest that the use of SIRC cell layers can reasonably predict the uptake of biotin and riboflavin across corneal epithelial membranes.[12,15] SIRC cell line was selected for characterization studies as it exhibits physiological and biochemical properties of rabbit corneal epithelium. Hence it could serve as a better *in vitro* model to study the folate uptake in rabbit cornea.[16] SIRC cell line has been widely utilized as a model for investigating transport of drugs across rabbit cornea. In our study, [^{3}H]Folic acid (10 nM) uptake was found to be saturable with an apparent K_m of 14.22 nM, V_{max} of $1.5*10^{-5}$ micromoles/min/mg protein and K_d of $2.10*10^{-6}$ $min^{-1}$for folic acid. Existence of different folic acid uptake mechanisms at varying levels of expression depends on cell lines and/or inherent gene sequence of that specific cell line. A common trend observed with folic acid uptake mechanism appears to be specific and saturable process in the range of nanomolar concentration (10–50 nM) suggesting the involvement of FR in the uptake of folic acid in SIRC cells.

Uptake process of folic acid was found to be time dependent and saturation in the uptake was observed after 30 min. The process is pH independent with maximum rate observed at a low pH i.e. pH 4 and 5. There was a significant decrease in the uptake with increase in the pH i.e. at 6, 7 and 8. This result clearly suggests proton coupled uptake of folic acid into SIRC cells. The process appears to be temperature dependent with optimal uptake at a physiological temperature of 37°C. At room temperature there was a significant decrease in the uptake. The process drastically reduced at 4°C, which clearly suggests the existence of a receptor. These observations were consistent with the previously published results from our laboratory with retinoblastoma (Y-79) cells.[13] Presence of chloride and sodium-free buffers caused considerable inhibition of [^{3}H]Folic acid uptake. This implies that chloride and sodium ions are involved in folic acid translocation. Additional support for Na^+ dependence has been shown through uptake studies performed in the presence of ouabain, a well-known Na^+/K^+-ATPase inhibitor. Significant decrease in folic acid uptake was observed in the presence of ouabain, suggesting that carrier-mediated transport is energy dependent. To determine whether the uptake is dependent on a motive energy force, known metabolic inhibitors (sodium azide and 2, 4-dinitrophenol) were added to the incubation media. Significant inhibition of [^{3}H]Folic acid uptake was observed when cells were treated with sodium azide (1 mM) and 2, 4-dinitrophenol (1 mM), which is known to reduce intracellular ATP. Thus, process of folic acid uptake in SIRC cells is found to be energy dependent and appears to be directly coupled to ATP energy sources. These results clearly indicate the involvement of a specialized, energy, sodium and chloride dependent and high-affinity carrier-mediated system which saturates at nanomolar concentrations. Possible involvement of a high affinity FR in the folic acid uptake was further supported by data showing a significant inhibition in the presence of unlabelled folic acid, MTF and MTX.

No significant inhibition in folic acid uptake was observed in the presence of various unlabelled vitamins (biotin, pantothenic acid, riboflavin, niacin and ascorbic acid).Taken together; these results provide an additional support for the presence of a carrier system that specifically mediates the uptake of folic acid into SIRC cells at nanomolar concentrations. Significant inhibition by these anion exchange inhibitors suggests the possible involvement

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of an anion-exchange transport mechanism probably through proton coupled folate transporter. However colchicine significantly reduced the folic acid uptake, suggesting the involvement of receptor mediated endocytosis process. Further studies are required to corroborate these findings. RT-PCR analysis provided evidence on the molecular expression of folate receptor and PCFT transporter. Therefore this data further supports the existence of a specific transport system for folic acid in SIRC cells. Clear distinct bands in Western bolt confirmed the presence of FR-α (40 kDa) and PCFT (50 kDa) protein expressions in SIRC. FR-α and PCFT proteins are involved in the influx of folic acid into corneal cells (SIRC). Various studies have demonstrated the regulation in activity of membrane transporter systems by the major signaling pathways, i.e., protein tyrosine kinase, protein kinase A^-, C^-, and Ca^{2+}/calmodulin-mediate pathways.[17] We also investigated possible regulation of the folic acid uptake process by intracellular regulatory pathways. Concentration dependent inhibition by TFP, KN-62, and calmidazolium suggest the involvement of Ca^{2+}/calmodulin mediated pathways in the regulation of folic acid uptake. PKC pathway modulators (PMA and bisindolylmaleimide I) did not cause any change in the uptake of folic acid in SIRC cells. However PKA pathway modulators (IBMX, forskolin and H-89) played an important role in the regulation of uptake. There was a significant lowering of uptake in the presence of PTK pathway modulators. Summarizing, from this work results indicate the involvement of a Ca^{2+}/calmodulin, PKA and PTK pathway but non-involvement of PKC pathways in the regulation of folic acid uptake. Physiological mechanism(s) through which calmidazolium and protein kinase pathways exert its regulatory effect on folic acid uptake is not clear. Most signal transduction pathways are involved in diverse and critical cellular functions. Physiological significance behind multiple-signal regulating pathways involved in folic acid uptake in cornea is poorly understood. Extensive cross-signaling between cAMP and calmodulin-mediated signal transduction pathways exists at several levels of cellular control. Therefore, calmodulin mediated reduction of folic acid uptake in SIRC cells might be a manifestation of intertwined regulation of these processes. In conclusion, this is the first report indicating the functional and molecular expression of FR and PCFT on SIRC cells. This carrier-mediated active transporter system is temperature, energy, sodium and chloride ion dependent. The process appears to be regulated by PKA, PTK and Ca^{2+}/calmodulin-mediated pathways. The existence of folate carrier mediated system was further confirmed by conducting *ex vivo* transport studies across rabbit cornea. This study also provides useful information on the substrate specificity of carrier system. SIRC cells may serve as a useful *in vitro* experimental model of rabbit cornea for delineating epithelium uptake mechanism and intracellular translocation of various anti-folate agents. Moreover, folate carrier-mediated system can be targeted through design of folate-conjugated prodrugs and nanoparticles to achieve enhanced permeability which may result in significant improvement in therapeutic outcomes for corneal diseases.

Conclusion

This research article suggests that SIRC cells may serve as a practical *in vitro* experimental model for rabbit cornea for delineating corneal uptake mechanisms. Folate carrier-mediated system can be utilized for targeted drug delivery to cornea using folate-conjugated nanoparticles and prodrugs to attain enhanced permeability which can result in significant improvement in therapeutic outcome of several corneal diseases.

Acknowledgments

This research was supported by Missouri Life Sciences Grant and NIH/NEI - 2R01 EY 10659 and NIH/NEI - 2R01 012830 grants.

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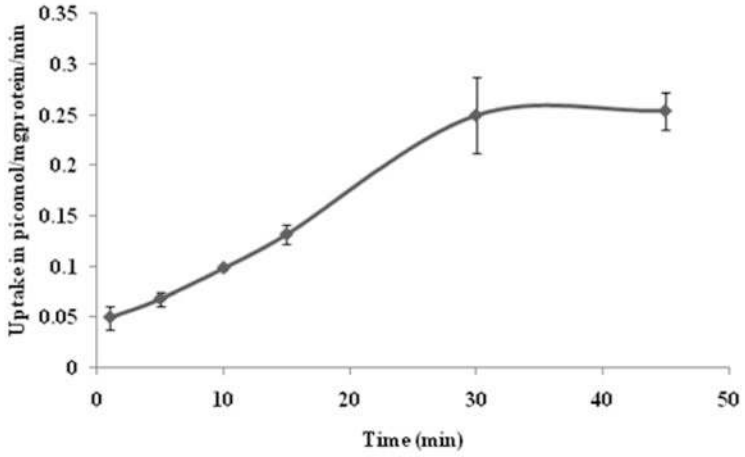

Fig. 1.

Uptake of [^{3}H]Folic acid by SIRC cells as a function of time. Each data point represents the mean α standard deviation of 5 separate uptake determinations.

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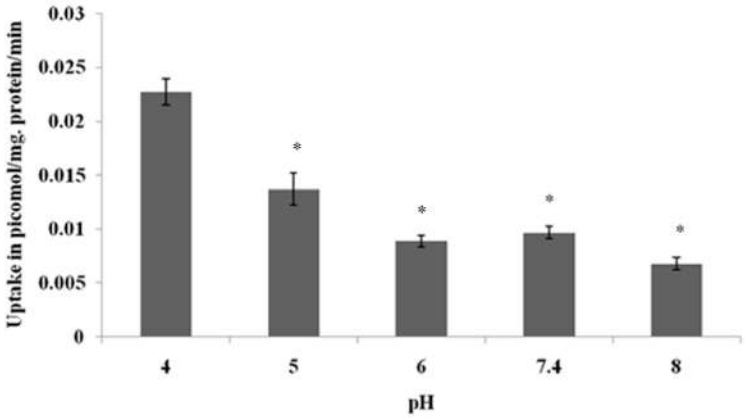

Fig. 2.

Uptake of [^{3}H]Folic acid by SIRC cells as a function of pH. Each data point represents the mean ± standard deviation of 5 separate uptake determinations. Asterisk (*) represents significant difference from control ($p < 0.05$).

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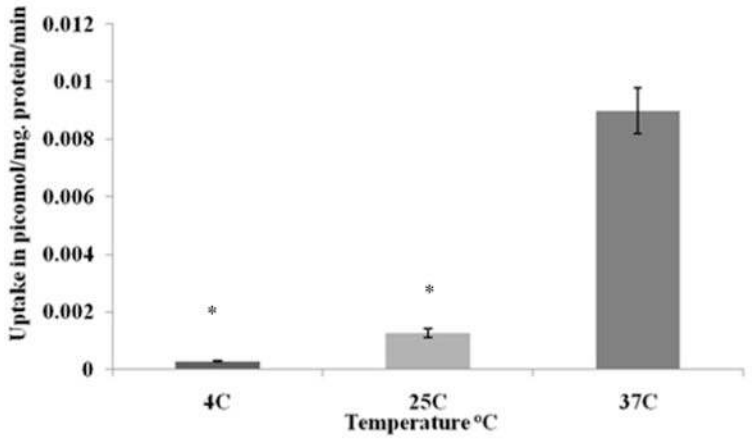

Fig. 3.

Uptake of [^{3}H]Folic acid by SIRC cells as a function of temperature. Each data point represents the mean ± standard deviation of 5 separate uptake determinations. Asterisk (*) represents significant difference from control ($p < 0.05$).

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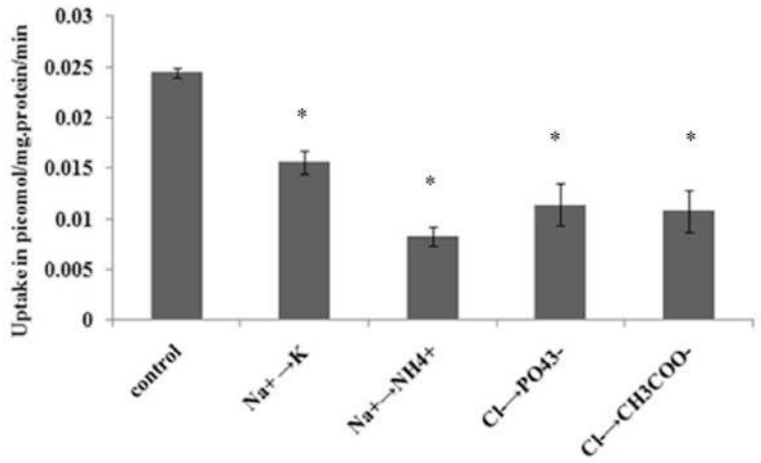

Fig. 4.

Uptake of [^{3}H]Folic acid by SIRC cells as a function of ions. Each data point represents the mean ± standard deviation of 5 separate uptake determinations. Asterisk (*) represents significant difference from control ($p < 0.05$).

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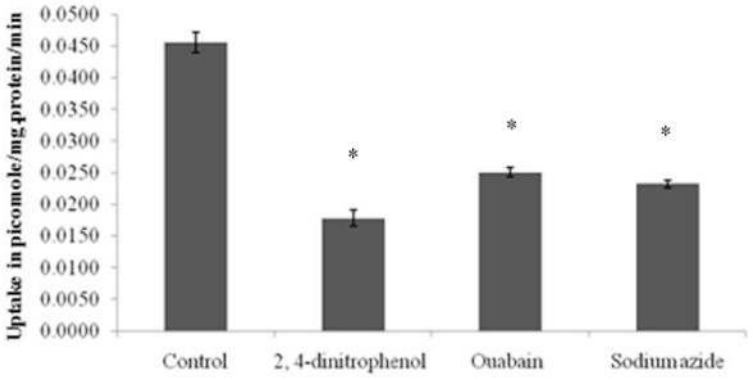

Fig. 5.

Uptake of [^{3}H]Folic acid by SIRC cells in presence of various energy inhibitors. Each data point represents the mean ± standard deviation of 5 separate uptake determinations. Asterisk (*) represents significant difference from control ($p < 0.05$).

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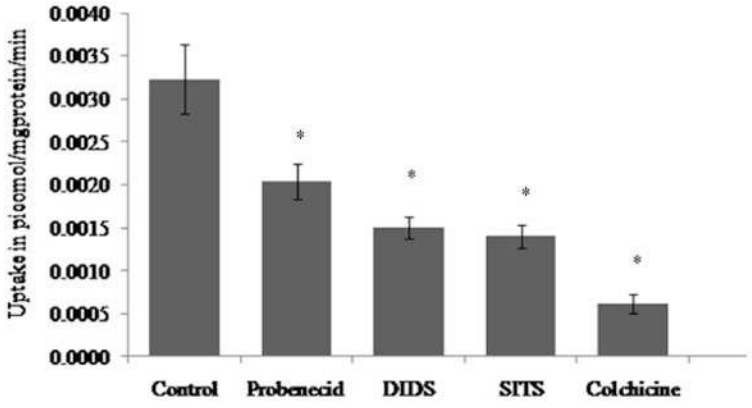

Fig. 6.

Uptake of [^{3}H]Folic acid by SIRC in presence of membrane transport inhibitors and endocytosis inhibitor colchicine. Each data point represents the mean α standard deviation of 5 separate uptake determinations. Asterisk (*) represents significant difference from control ($p < 0.05$).

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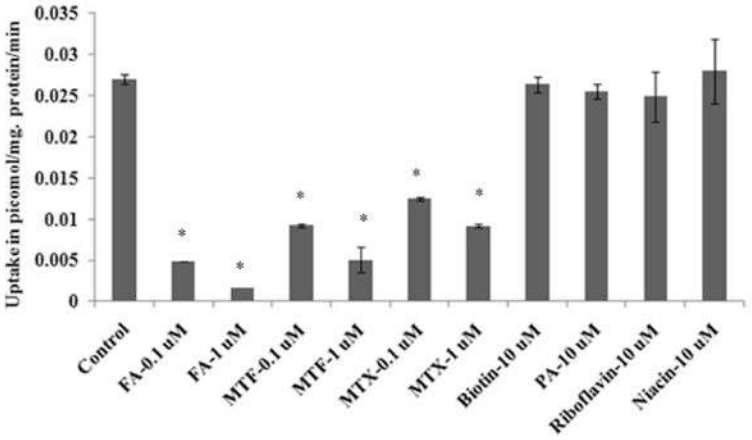

Fig. 7.

Substrate specificity of uptake of [^{3}H]Folic acid (10 nM) by SIRC cells in presence of various structurally related and unrelated analogs. Each data point represents the mean standard deviation of 5 separate uptake determinations. Asterisk (*) represents significant difference from control ($p < 0.05$).

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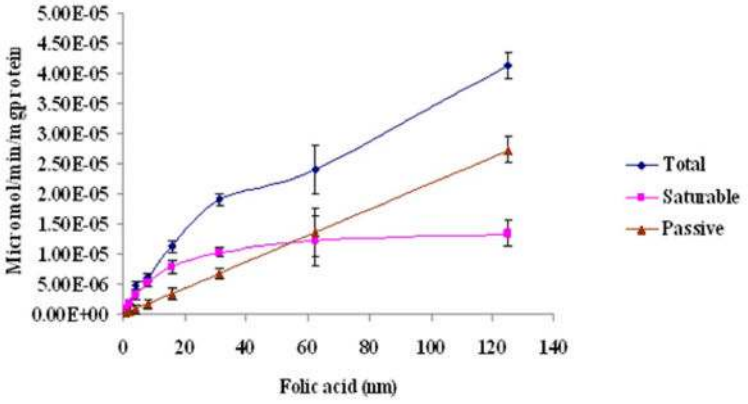

Fig. 8.

Uptake of [^{3}H]Folic acid (10 nM) in presence of various concentrations of cold folic acid on SIRC cell line.Saturation kinetic parameters are as follows : V_{max}: $1.5*10^{-5}$, K_m : 14.22 nM and K_d: $2.1*10^{-6}$ min.$^{-1}$ Each data point represents the mean standard deviation of 5 separate uptake determinations.

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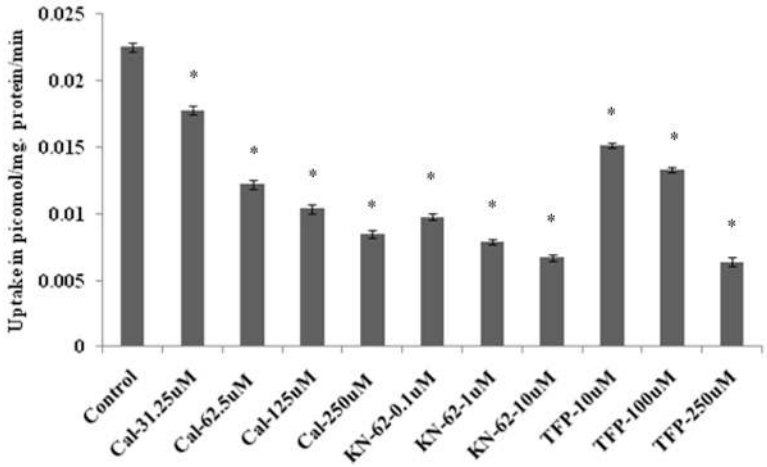

Fig. 9.

Effect of Ca^{2+}/calmodulin-mediated pathways modulators on the uptake of [^{3}H]Folic acid in SIRC cells. Each data point represents the mean standard deviation of 5 separate uptake determinations. Asterisk (*) represents significant difference from control ($p < 0.05$).

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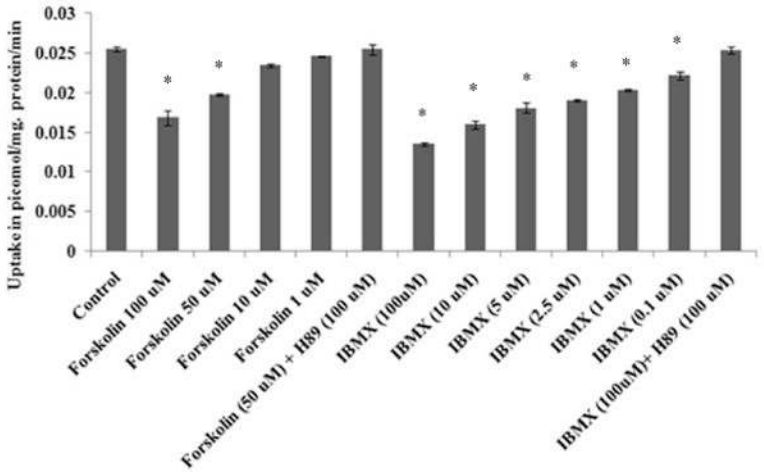

Fig. 10.

Effect of PKA pathway modulators on the uptake of [^{3}H]Folic acid in SIRC cells. Each data point represents the mean standard deviation of 5 separate uptake determinations. Asterisk (*) represents significant difference from control ($p < 0.05$).

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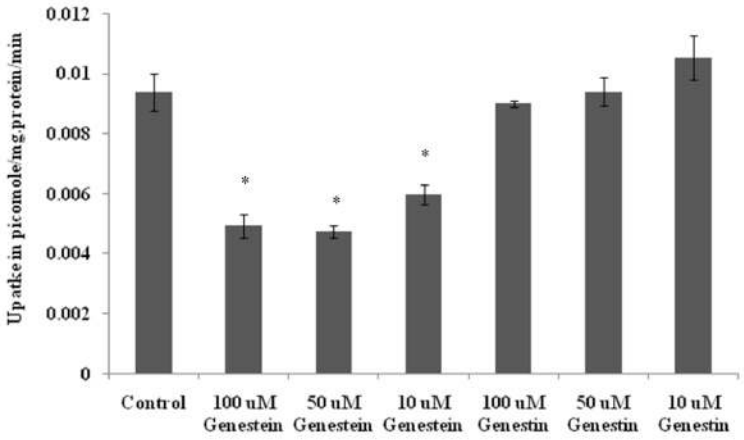

Fig. 11.

Effect of PTK pathway modulators on the uptake of [^{3}H]Folic acid in SIRC cells. Each data point represents the mean standard deviation of 5 separate uptake determinations. Asterisk (*) represents significant difference from control ($p < 0.05$).

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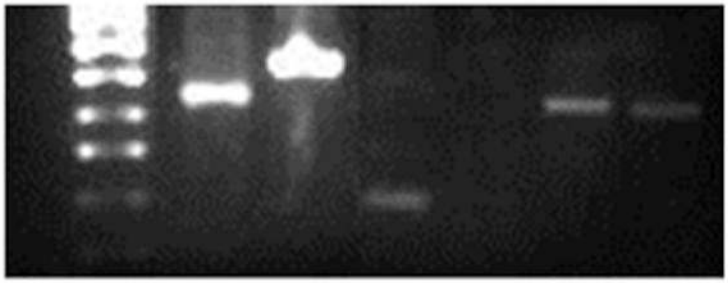

Fig. 12.
RT-PCR analysis of FR-α (Folate receptor), RFC (reduce folate carrier), PCFT (Proton coupled folate transporter). GAPDH (Glyceraldehyde 3-phosphate dehydrogenase)

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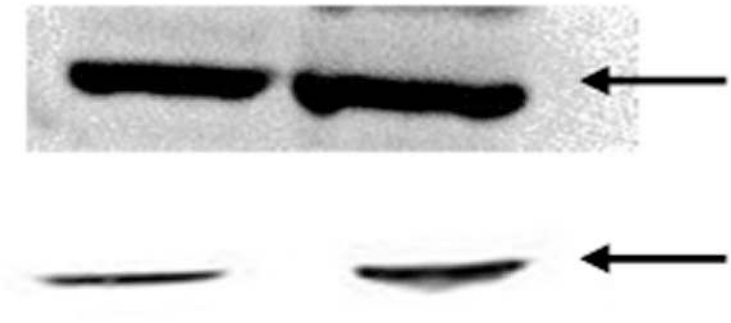

Fig. 13.
Western blot analysis of FR-α (Folate receptor) and PCFT (Proton coupled folate transporter).

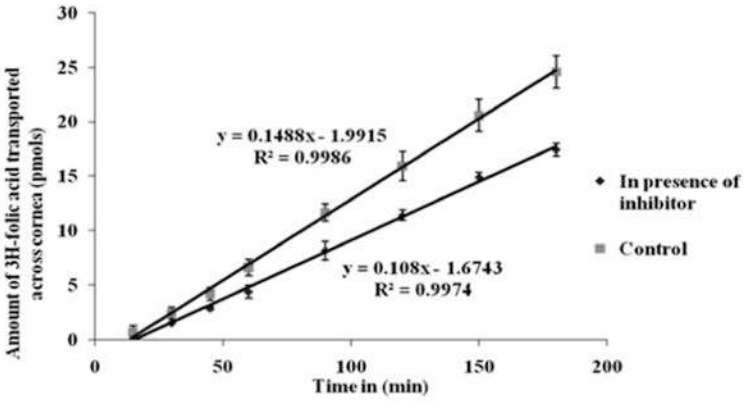

Fig. 14.

Ex vivo permeability of [^{3}H]Folic acid in excised rabbit cornea. Each data point represents the mean standard deviation of 4 separate determinations.

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Table 1

Primers for RT-PCR analysis

Gene	NCBI Accession code	Sequence (5′->3′)	Product length
GAPDH	NM_002046.3	Forward : GGGAAGGTGAAGTTCGGAGT	633
		Reverse : GCCAGTAGAGGCAGGGATGA	
GAPDH	NM_002046.3	Forward : GTCCACCACTGACACGTTGG	729
		Reverse : GGGAAGGTGAAGGTCGGAGT	
FRα	NM_016724.2	Forward : GCATTTCATCCAGGACACCT	407
		Reverse : TCATGGCTGCAGCATAGAAC	
RFC	NM_194255.1	Forward : GCTCCTACCAGTTCCTCGTG	621
		Reverse : AGACACTGCAAACCCAGCTT	
PCFT	NM_080669.3	Forward : CTCCACGTCGGCTACTTCGT	625
		Reverse : CCATCCCCAGGATGTTGAAG	
PCFT	NM_080669.3	Forward : CTCCAGGTCGGCTACTTCGT	624
		Reverse : CATCCCCAGGATGTTGAAGG	

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Table 2

Ex vivo

permeability of [^{3}H]Folic acid in excised rabbit cornea

Sample	Permeability (cm/sec)* 10^5 (mean ±SEM)
Control ([^{3}H]Folic acid)	1.48± 0.13
In presence of folic acid	1.08± 0.10