Original Article

Functional characterization and expression of folate receptor-α in T47D human breast cancer cells

Abstract

Purpose: The objective of this study was to investigate the functional and molecular expression of a carrier mediated system responsible for folate uptake in breast cancer (BC) (T47D) cells and to delineate the mechanism of intracellular regulation of this transport system. **Materials and Methods:** [^{3}H]-folic acid uptake was studied in T47D cells with respect to time, pH, temperature, sodium and chloride ion dependency. Inhibition studies were conducted in the presence structural analogs, vitamins, metabolic and membrane transport 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; calcium-calmodulin, protein kinases A and C (PKA and PKC) and protein tyrosine kinase (PTK). Molecular evidence was studied by qualitative and quantitative polymerase chain reaction (PCR) and Western blot analysis. **Results:** Linear increase in [^{3}H]-folic acid uptake was observed over 30 min. The process followed saturation kinetics with an apparent K_m of 11.05 nM, V_{max} of 1.54 * 10^{-8} μmoles/min/mg proteins and K_d of 9.71 * 10^{-6}/min for folic acid. 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, methyltetrahydro folate and methotrexate), but structurally unrelated vitamins did not show any effect. Membrane transport inhibitors such as SITC, DIDS, probenecid and endocytic inhibitor colchicine significantly inhibited the [^{3}H]-folic acid uptake process. PKA, PTK and Ca^{2+}/calmodulin pathways positively regulate the uptake process. Reverse transcriptase polymerase chain reaction (RT PCR) analysis had shown mRNA expression of folate receptor (FR)-α at 407 bp. Quantitative polymerase chain reaction analysis showed significantly higher FR-α mRNA levels in T47D cells compared to MCF-7 cells and Western blot analysis confirmed the FR-α protein expression at 37 kDa. **Conclusions:** This work demonstrated the functional characterization and molecular presence of FR-α in the T47D cell line. The high expression of FRs in T47D human breast carcinoma cells supports their validity as molecular therapeutic targets in BC.

Key words:
Breast cancer cells, folate receptor-α, T47D cells

Introduction

Breast cancer (BC) is one of the most commonly observed malignancies in women.[1] BC is usually treated with surgery followed by chemotherapy or radiation therapy, or both. Chemotherapeutic agents generally work by destroying the fast-growing cancer cells either by damaging DNA upon replication or other mechanisms. Multiple drug resistance (MDR) in BC cells is associated with the over expression of efflux pumps. MDR is a phenomenon where tumor cells develop resistance to a wide range of structurally and functionally unrelated therapeutic agents. MDR genes produce three main types of efflux proteins: P-glycoprotein, multidrug resistance associated proteins and BC resistance

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protein. These efflux proteins play a critical role in protecting cells against cytotoxic drugs. Folate compounds, including folate analogs, are internalized by mammalian cells through either carrier-mediated[2-4] or receptor-mediated[2,5] processes. The former binds to a mobile carrier at the plasma membrane surface and from there mediates bidirectional flux. The later binds with a much higher affinity, to a receptor protein at the membrane surface causing it to more slowly mediate unidirectional flux. This occurs after internalization of the receptor-folate compound complex in the manner of many other receptor-ligand processes.[6,7] Folate receptors (FRs) are membrane-bound glycoproteins of 32-36 kDa with a very high affinity for folic acid and its structural analogs methyltetrahydrofolate (MTF) and methotrexate (MTX).[8] FRs are clustered on the cell surface and are associated with uncoated membrane invaginations (caveolae). They are considered to be vital components in maintaining the mammalian cellular folate homeostasis. FRs are generally coded by two genes (FR-α and -β) with differential tissue expression.[9] FR regulates the uptake of folic acid by a process known as potocytosis.[7] They move in and out of the cell through a new uncoated pit pathway that does not involve the clathrin-coated pit endocytic machinery. Initially, folate binds to the outwardly oriented FR and then the internalization of caveolae takes place with the formation of a compartment that is acidified by proton pump.[10]

Folate conjugated drug delivery systems such as nanoparticles, liposomes and micelles expand the therapeutic window of various anticancer drugs by augmenting delivery to the target tissue. Selective targeting not only reduces the minimum effective dose and toxicity of chemotherapeutic agents, but also enhances their therapeutic efficacy with equivalent plasma concentrations.[11,12] Folate as a targeting moiety offers greater advantages than macromolecules such as antibodies. These include: (a) Miniature size of the targeting moiety, which offers favorable pharmacokinetic properties of folate conjugates and decreases the probability of immunological reactions allowing for repeated administration, (b) very low cost and availability, (c) high affinity for receptors, (d) the receptor-ligand conjugates can be internalized by the cell through endocytosis, which aids in cytosolic delivery of therapeutic agents and (e) high expression among BC cell lines offer a great potential for future therapeutic and diagnostic applications in treating BC patients.[13] The over expression of FR, by a variety of tumor cell lines may present an appropriate mechanism for targeted delivery of radiopharmaceuticals to tumors for diagnostic imaging and treatment of various malignancies.[14] The FR is a folate binding protein that binds to folate and folate-conjugated drugs with high affinity (K_d ~ 100 pM).[15] The alpha isoform of the FR-α is a well-known tumor marker, with prominent expression in numerous epithelial cancers, but partial expression in normal tissues.[16] The healthy tissues which express FR-α include the proximal tubules of kidney, epithelial cells of the lung, intestine and choroid plexus.[17] FR-α is up-regulated across the entire plasma membrane by a large range of epithelial cancers.[17] Those epithelial cancers known to over express FR-α include cancers of the ovary, lung, kidney, head, retina and neck.[17-20]

Human BC (T47D) cells are a hypotriploid human cell line derived from breast ductal carcinoma. The main purpose of selecting T47D cells as an *in vitro* cell culture model for our studies is due to their aggressive characteristics.[21] Expression of several transport systems such as glucose,[22] monocarboxylate[23] and cationic amino acid,[24] biotin[25] transporters have been reported in T47D cells. However, no previous work investigating the mechanistic and functional process of intracellular trafficking of folate uptake and molecular expression of FR-α in T47D cells has been reported. Therefore, the objective of this study was to investigate the functional and molecular expression of a carrier mediated system responsible for folate uptake on the epithelia of BC cells and to delineate its mechanism of uptake and intracellular regulation. Saturation kinetics of folate uptake in T47D cells was studied and K_m and V_{max} were determined. We further studied and compared the mRNA expression levels of FR-α by quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) in both T47D and MCF-7 cells.

Materials and Methods

Materials

[^{3}H]-folic acid (50 Ci/mmol) was purchased from Perkin-Elmer (Boston, MA). Unlabelled folic acid, MTF, MTX, biotin, ascorbic acid, riboflavin, pantothenic acid, sodium azide, ouabain, 2,4-dinitrophenol, PTK modulators (genistin and genistein), PK (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, 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), d-glucose and all other chemicals were procured from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals were of special reagent grade and used without further purification.

Cell culture

T47D cell line between passages 28 and 38 was utilized for these studies. All cultures were maintained in a humidified incubator at 3°C with 5% carbon dioxide in air atmosphere. The culture medium containing Dulbecco's modified Eagle's medium, 10% fetal bovine serum (heat inactivated), 1% non-essential amino acids, 4 mM l-glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin

and 14 mM HEPES at pH 7.4 was added according to the protocol established in our laboratory for maintaining cell cultures. At 80% confluence, cells were removed by treating with trypsin/ethylenediaminetetraacetic acid and 250,000 cells/cm^2 were plated on a 12-well tissue culture plastic plate for uptake studies. The culture medium was replaced every alternate day for a week. The monolayers were grown for 8-10 days after seeding were utilized for all uptake studies.

General procedure of uptake experiments

T47D cells were washed three times with 2 ml of Dulbecco's phosphate buffered saline (DPBS) buffer at 37°C for 10 min each time, unless otherwise stated. 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 the cells were incubated for 30 min. Following incubation, the cells were washed thrice with an ice-cold stop solution (200 mM KCl and 2 mM HEPES) to stop the folic acid uptake. Cells were lysed overnight (using 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 of scintillation cocktail (Fisher Scientific, Fair Lawn, NJ, USA). Samples were then analyzed by a liquid scintillation counter (Model LS-6500, Beckman Instruments, Inc., Fullerton, CA, USA). The amount of protein in the cell lysate was measured by BioRad Protein Estimation Kit (BioRad, Hercules, CA, USA) and the rate of uptake was normalized to the protein content of each well. Cell viability under all treatment conditions was monitored by Trypan blue exclusion test and was routinely observed to be between 90% and 95%.[8,26,27]

Time dependent cellular uptake for folic acid

Uptake of [^{3}H]-folic acid was studied at various time points (1, 2, 5, 10, 15, 30 and 45 min) in T47D cells to optimize the time required for carrying out further studies.

Effect of pH and temperature on the cellular uptake of folic acid

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 of [^{3}H]-folic acid, buffer temperatures were adjusted to 4°C, 25°C and 37°C. Uptake of [^{3}H]-folic acid (10 nM) in T47D cells was performed at varying pH and temperature conditions.

Effect of membrane transport inhibitors

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

Effect of energy modulators

To examine the energy dependence, T47D cells were pre-incubated with metabolic inhibitors (1 mM), like ouabain (an inhibitor of Na^+/K^+-ATPase), 2,4-dinitrophenol (intracellular adenosine triphosphate (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 ions

In order to investigate ion dependency, sodium ions in the bathing media were replaced with equimolar quantities of K^+, NH^{4+}, choline chloride and Cl – ions with salts of organic and inorganic monovalent ions (phosphate and acetate).

Substrate specificity

In order to describe the structural requirements for interaction with the folate carrier mediated system, uptake studies were carried out in the presence of various vitamins and structural analogs. The unlabeled vitamin or structural analog was concurrently 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 μM were employed in these studies.

Concentration dependent study data analysis

Uptake of [^{3}H]-folic acid was carried out in T47D cells in the 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 described by the Michaelis-Menten equation and a non-saturable passive diffusion process:

$$V = \frac{V_{max}[C]}{K_m + [C]} + K_d[C] \quad (1)$$

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

examined by 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 ± standard deviation. Michaelis-Menten parameters such as K_m, K_d and V_{max} are expressed as mean ± standard error. Unpaired Student's *t*-test was used to estimate statistical significance. A difference between mean values was considered as significant if $P < 0.05$.

Intracellular regulation of cellular PK-mediated pathways

Involvement of intracellular regulatory pathways such as PKC, PKA, 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 PK II inhibitor (KN-62). Solutions of these modulating agents were prepared in DMSO or absolute ethanol (final concentration of the organic solvent was less than 1%, v/v). Cells were preincubated for 1 h with various pathway 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 ribonucleic acid (RNA) was isolated from T47D cells using Trizol® reagent (Invitrogen) by a standard protocol. Briefly, cells were lysed by adding 800 µl of Trizol reagent. The lysate was then transferred to ependorff 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 M-MLV Reverse transcriptase (Promega, Madison, WI, USA). 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 using 1 µg cDNA and primers used for the amplification were shown in Table 1. GAPDH was used as an 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 gel electrophoresis using 1.5% agarose gel electrophoresis and visualized by chemiluminescence.

Gene expression – quantitative analysis

Total RNA was extracted from the T47D cells using TRIzol™ reagent (Invitrogen) following the manufacturer's protocol. RNA was suspended in DNase-RNase free water and concentration was determined. 2 µg of total RNA was reverse transcribed to obtain first strand cDNA using oligodT and GoScript™ Reverse Transcription System (Promega). A volume of 3 µl of cDNA was then subjected to PCR using primers specific for the human FR. The conditions for PCR are initial denaturation at 94°C for 45 s, annealing at 58°C for 1 min and extension at 72°C for 45 s for a total of 35 amplification cycles, followed by a final extension of 72°C for 10 min. The product was subjected to gel electrophoresis using 3% agarose containing ethidium bromide and visualized under UV. Quantitative real time PCR was performed to compare the FR expression levels in T47D and MCF-7. RNA was extracted using the above mentioned procedure. Quantitative real time PCR was performed using Lightcycler SYBR-green technology (Roche) using cDNA equivalent to 80 ng of total RNA. PCR products were subjected to a melting-curve analysis to confirm the PCR specificity. The comparative threshold method was used to calculate the relative amount of RNA in T47D with MCF-7. The real time primers used for the study are summarized below in Table 2. GAPDH was used as an internal control in both cases.

Table 1: Primers for qualitative RT-PCR analysis

Gene	NCBI accession code	Sequence (5′- > 3′)	Product length
GAPDH	NM_002046.3	Forward: GGGAAGGTGAAGTTCGGAGT Reverse: GCCAGTAGAGGCAGGGATGA	633
FR-α	NM_016724.2	Forward: GCATTTCATCCAGGACACCT Reverse: TCATGGCTGCAGCATAGAAC	407
RFC	NM_194255.1	Forward: GCTCCTACCAGTTCCTCGTG Reverse: AGACACTGCAAACCCAGCTT	621
PCFT	NM_080669.3	Forward: CTCCACGTCGGCTACTTCGT Reverse: CCATCCCCAGGATGTTGAAG	625

RT-PCR – Reverse transcriptase-polymerase chain reaction; NCBI – National Center of Biotechnology Information; GAPDH – Glyceraldehyde 3-phosphate dehydrogenase; FR-α – Folate receptor-α; RFC – Reduce folate carrier; PCFT – Proton coupled folate transporter

Table 2: Primers for qPCR analysis of FR-α, mRNA expression in T47D cells compared to MCF-7 cells. Sequence is given from 5′->3′

	Primers
GAPDH	
Forward	AACAGCGACACCCACTCCTC
Reverse	ACCACCCTGTTGCTGTAGCC
FR-α	
Forward	AGGACAGACATGGCTCAGCG
Reverse	TGTGGTGCTTGGCGTTCATG

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase; FR-α – Folate receptor-α; qPCR – Quantitative polymerase chain reaction; mRNA – Micro-ribonucleic acid

Computer analysis

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

Western blot analysis for folate transport proteins

Protein was extracted from T47D 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 s followed by centrifugation at 8000*g* for 15 min. Protein samples were subjected to sodium dodecyl sulfate poly-acrylamide gel electrophoresis and after the transfer to nitrocellulose membranes, were incubated with antibody against FR-α, proton coupled folate transporter (PCFT), or reduced folate carrier (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 expression was visualized with the Super Signal West Pico Chemiluminescence Detection System (Thermo Scientific, Rockford, IL, USA). β-Actin served as a loading control.

Results

Time dependent uptake

Time-dependent uptake of [^{3}H]-folic acid (10 nM) was carried out in T47D cells. As shown in Figure 1, the uptake increased linearly up to 30 min and reached equilibrium at 45 min. Hence, all uptake experiments were carried out over 30 min unless otherwise mentioned.

pH dependence

Role of hydrogen ions on the uptake of [^{3}H]-folic acid was examined by varying 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 [Figure 2]. Since the uptake of folic acid 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°C, 25°C and 37°C). As indicated in Figure 3, the rate of uptake significantly reduced at 4°C 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 of 37°C.

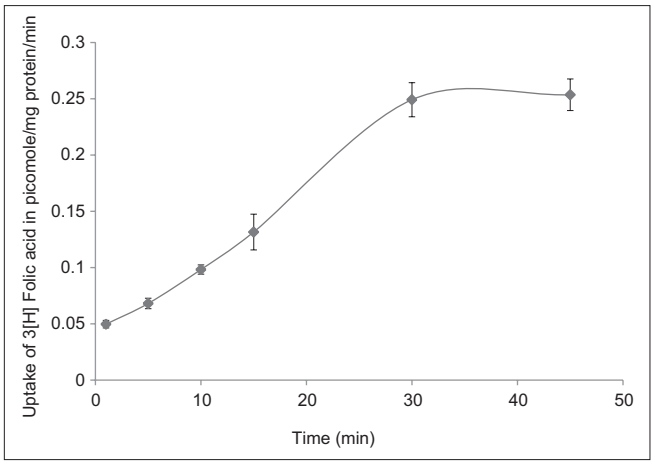

Figure 1: Uptake of [^{3}H]-folic acid by T47D cells as a function of time. Each data point represents the mean ± standard deviation of five separate uptake determinations

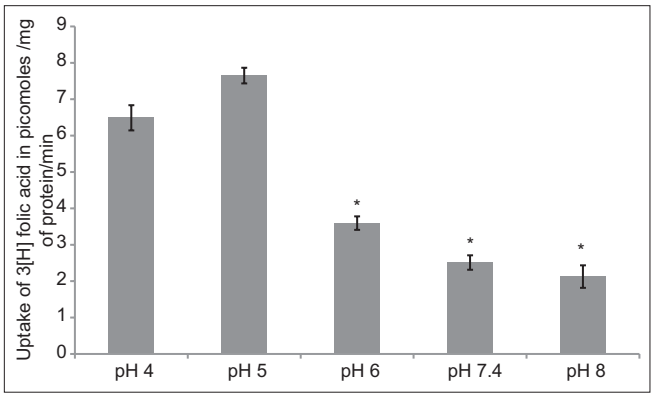

Figure 2: Uptake of [^{3}H]-folic acid by T47D cells as a function of pH. Each data point represents the mean ± standard deviation of five separate uptake determinations. Asterisk (*) represents a significant difference from control ($P < 0.05$)

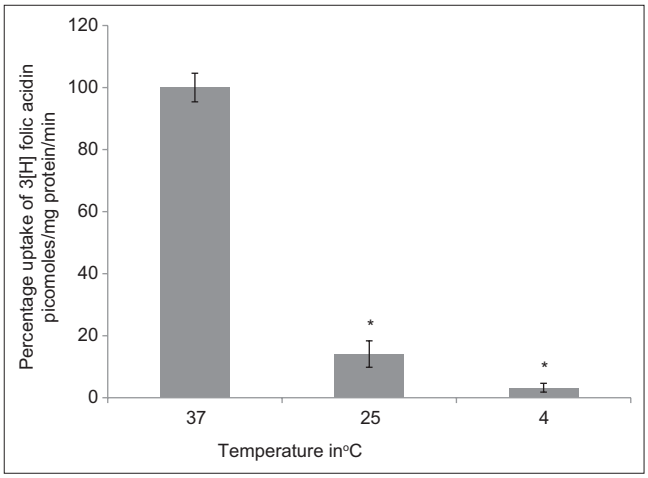

Figure 3: Uptake of [^{3}H]-folic acid by T47D cells as a function of temperature. Each data point represents the mean ± standard deviation of five separate uptake determinations. Asterisk (*) represents a significant difference from control ($P < 0.05$)

Role of membrane transport inhibitors

The role of membrane transporter inhibitors was investigated by incubating the cells with probenecid, DIDS and SITC and endocytosis process inhibitor colchicine at 1 mM concentration. As indicated in Figure 4, there was a significant decrease 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 the presence of 1 mM colchicines and cytochalasin suggests the involvement of receptor mediated endocytosis. This result suggests the existence of receptors in the T47D cell line.

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. Figure 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 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 6 clearly illustrates Na^+ and Cl^- ion dependency of folate uptake process in T47D cells.

Concentration dependency

The existence of a carrier-mediated system for folic acid in the T47D 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 illustrate 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 of folate [Figure 7]. Saturable as well as non-saturable components were determined by substituting the values of the kinetic constants into the 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 a modified Michaelis-Menten equation, an uptake process with apparent K_m of 11.05 nM, V_{max} of 1.54 * 10^{-8} μmoles/min/mg protein and K_d of 9.71 * 10^{-6}/min for folic acid 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

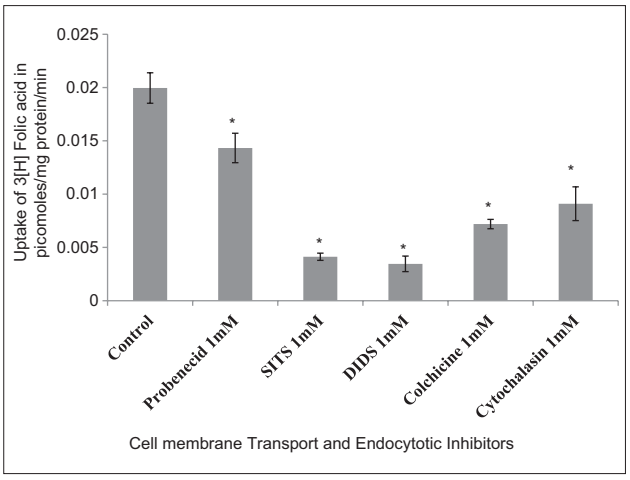

Figure 4: Uptake of [^{3}H]-folic acid by T47D cells in the presence of membrane transport inhibitors and endocytosis inhibitors. Each data point represents the mean ± standard deviation of five separate uptake determinations. Asterisk (*) represents a significant difference from control ($P < 0.05$)

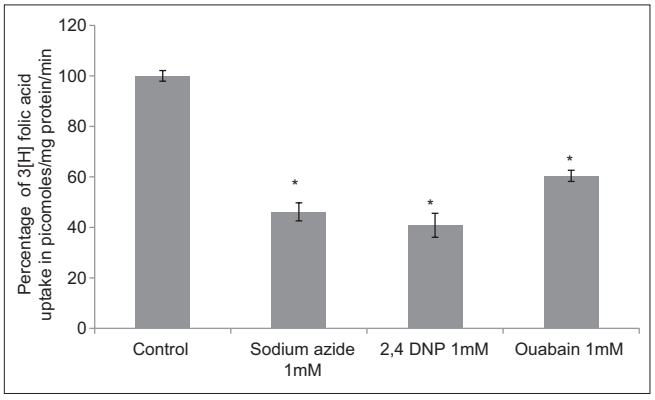

Figure 5: Uptake of [^{3}H]-folic acid by T47D cells in the presence of various energy inhibitors. Each data point represents the mean ± standard deviation of five separate uptake determinations. Asterisk (*) represents a significant difference from control ($P < 0.05$)

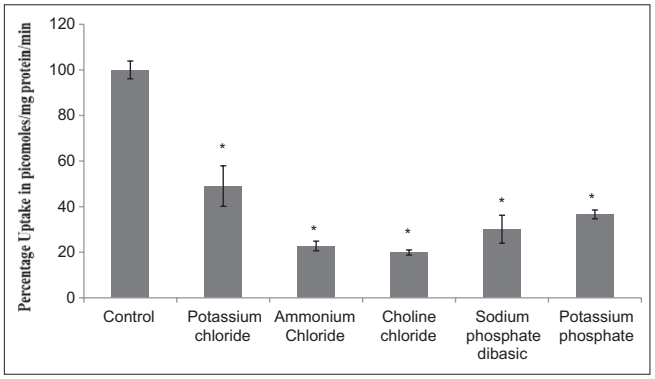

Figure 6: Uptake of [^{3}H]-folic acid by T47D cells as a function of ions. Each data point represents the mean ± standard deviation of five separate uptake determinations. Asterisk (*) represents a significant difference from control ($P < 0.05$)

in T47D cells by pre-treating them with calmodulin inhibitors (calmidazolium and tri-fluperazine) and with Ca^{2+}/calmodulin dependent PK II inhibitor (KN-62). These compounds significantly ($P < 0.05$) reduced the folic acid uptake in a concentration dependent manner as shown in Figure 8. Role of PKA-mediated pathway in the regulation of folic acid uptake was also studied by pre-treating T47D cells for 1 h with compounds that are known to increase intracellular cyclic adenosine monophosphate (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 Figure 9. Reduction in folic acid uptake by cAMP modulators suggested the involvement of cAMP-dependent 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 enzyme inhibitor, genistein. Genistin is considered a negative control for the PTK enzyme inhibitor. There was a significant difference in the uptake of [^{3}H]-folic acid in the presence of genistein (10-100 μM) as shown in Figure 10. These results suggest that Ca^{2+}/calmodulin, PKA and PTK pathways play a significant role in the translocation of folic acid into T47D cells.

Molecular evidence

Qualitative (RT-PCR) analysis

Expression of the folate carrier systems in T47D cells at the mRNA level was determined by RT-PCR analysis [Figure 11]. Gel electrophoresis had shown a major band (407 bp) corresponding to the amplified FR-α (FR-α: Lane 3). No product was observed for reduced folate carrier (RFC: Lane 4). Similarly, no band was observed for proton coupled folate transporter (PCFT: Lane 5). BLAST analysis showed that the primers used in this study can result in a PCR product size as specified.

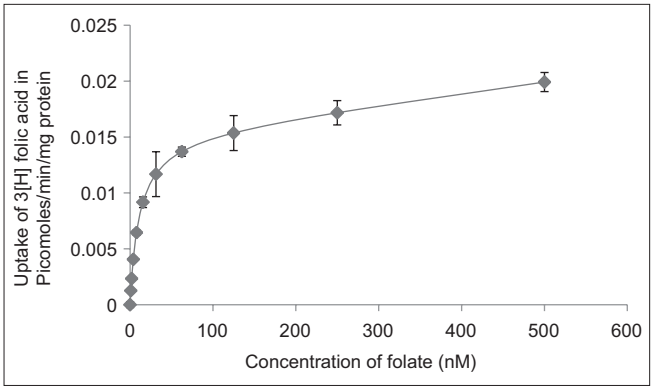

Figure 7: Uptake of [^{3}H]-folic acid (10 nM) in the presence of various concentrations of cold folic acid in T47D cells. Saturation kinetic parameters are as follows: K_m of 11.05 nM, V_{max} of 1.54 * 10^{-8} μmoles/min/mg protein and K_d of 9.71 * 10^{-6}/min for folic acid. Each data point represents the mean ± standard deviation of five separate uptake determinations

Quantitative (qPCR) analysis

Quantitative real time PCR [Figure 12] study confirms the high mRNA expression level of FR-α in T47D cells (approximately 13 fold) compared with MCF-7 cells [Table 3].

Western blot analysis

Western blot analysis [Figure 13] indicated the expression of FR-α protein at 37 kDa molecular weight and a clear distinct band was observed. Hence, this result confirms the existence of FR-α in T47D cells. Western blot analysis did not show any expression for RFC and PCFT proteins.

Discussion

The aim of this study was to identify a folate carrier mediated system in the T47D human BC cell line and to evaluate it as an *in vitro* screening tool for regulating the mechanism and intracellular regulation of folic acid uptake. T47D cell line was selected for characterization studies as it is derived from human BC. Hence, it could serve as a better *in vitro* model to study the folate uptake in human BC tissue. T47D cell line has been widely utilized as a model for investigating the targetability of various imaging agents

Table 3: Relative expression of FR-α in T47D and MCF-7 cells

	Relative fold expression	Standard error
T47D	13.8922571	0.51718
MCF-7	1	0.114891

FR-α – Folate receptor-α

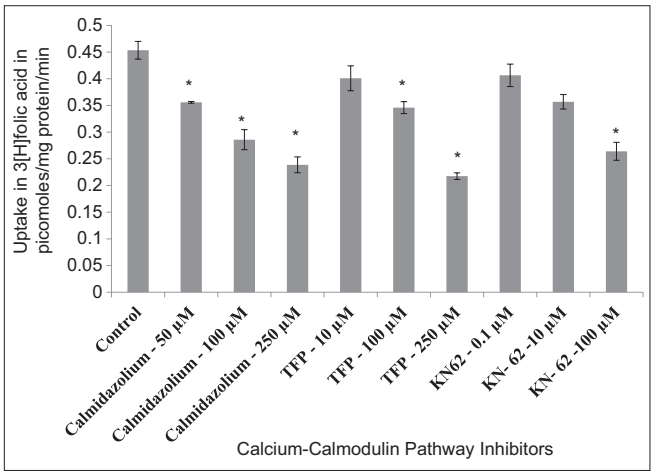

Figure 8: Effect of Ca^{2+}/calmodulin-mediated pathways modulators on the uptake of [^{3}H]-folic acid in T47D cells. Each data point represents the mean ± standard deviation of five separate uptake determinations. Asterisk (*) represents a significant difference from control ($P < 0.05$)

to detect BC tumors. In our study, [^{3}H]-folic acid (10 nM) uptake was found to be saturable with an apparent K_m of 11.05 nM, V_{max} of 1.54 * 10^{-8} μmoles/min/mg protein and K_d of 9.71 * 10^{-6}/min for folic acid. The 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 the folic acid uptake mechanism appears to be specific and saturable at nanomolar concentration (10-50 nM). This suggests the involvement of FR in the uptake of folic acid in T47D cells. The 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 dependent with the maximum rate observed at a low pH, i.e., pH 4 and 5. There was a significant decrease in the uptake with an increase in the pH, i.e., at 6, 7 and 8. This result clearly suggests the uptake of folic acid into T47D cells was mediated by protons. The process appears to be temperature dependent with an optimal uptake at 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. 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 the 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 energy, 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,

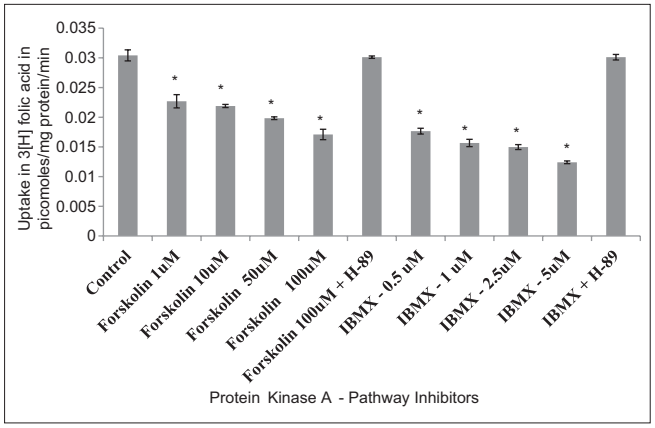

Figure 9: Effect of PKA pathway modulators on the uptake of [^{3}H]-folic acid in T47D cells. Each data point represents the mean ± standard deviation of five separate uptake determinations. Asterisk (*) represents a significant difference from control ($P < 0.05$)

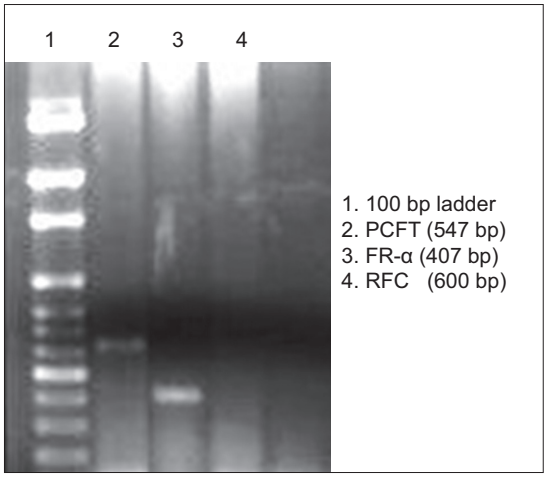

Figure 11: Qualitative reverse transcriptase-polymerase chain reaction analysis of folate receptor-α, reduce folate carrier, proton coupled folate transporter, glyceraldehyde 3-phosphate dehydrogenase, 1. 100 bp ladder, 2. PCFT (547 bp), 3. FR-α (407 bp), 4. RFC (600 bp)

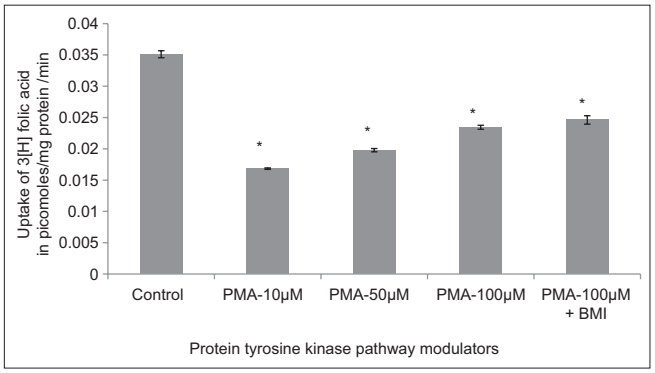

Figure 10: Effect of protein tyrosine kinase pathway modulators on the uptake of [^{3}H]-folic acid in T47D cells. Each data point represents the mean ± standard deviation of five separate uptake determinations. Asterisk (*) represents a significant difference from control ($P < 0.05$)

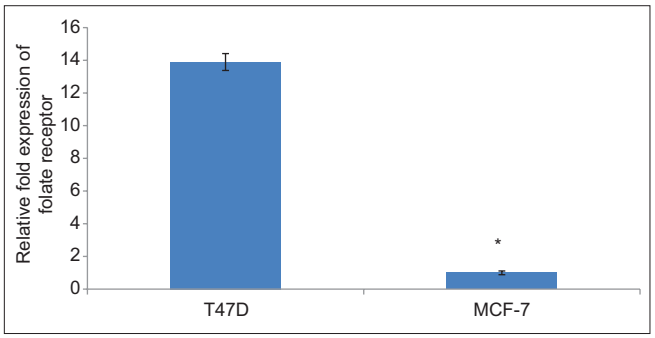

Figure 12: Quantitative real time polymerase chain reaction analysis of FR-α (FR) in T47D cells and MCF-7 cells

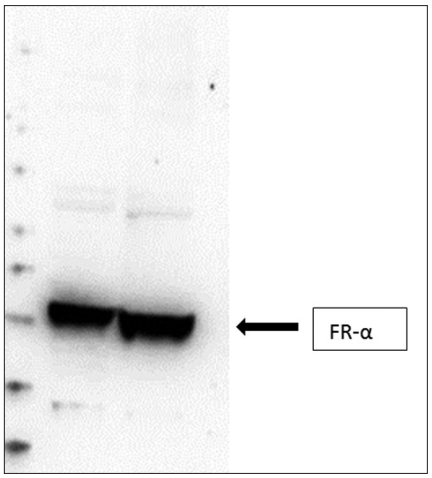

Figure 13: Protein expression by Western blot analysis of folate receptor (FR)-α and -β actin in T47D cells

the process of folic acid uptake in T47D cells was found to be energy dependent and appeared 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. The possible involvement of a high affinity FR in the folic acid uptake was further supported by data showing significant inhibition in the presence of unlabeled folic acid, MTF and MTX. No significant inhibition in folic acid uptake was observed in the presence of various unlabeled vitamins (biotin, pantothenic acid, riboflavin, niacin and ascorbic acid). Taken together; these results provide additional support for the presence of a carrier system that specifically mediates the uptake of folic acid into T47D cells at nanomolar concentrations. Significant inhibition was seen by the membrane transport inhibitor which suggests the possible involvement of an anion-exchange transport mechanism. However, colchicine and cytochalasin significantly reduced the folic acid uptake, suggesting the involvement of a receptor mediated endocytic process. Further studies are required to corroborate these findings. RT-PCR analysis provided evidence for the molecular expression of FR-α. Therefore, this data further supports the existence of a specific transport system for folic acid in T47D cells. The quantitative expression of FR-α at mRNA level in T47D cells was found to be seven times higher compared with MCF-7 cells. High expression of FR in T47D cells clearly indicates that T47D cell line serves as good *in vitro* cell culture model for studying the cellular accumulation of folate conjugated prodrugs and drug delivery systems. Western blot analysis showed a clear distinct band of 37 kDa upon incubation of T-47D cell protein with antibodies against FR-α indicating the gene encoding the FR-α protein is also expressed on in T47D cells. Various studies have demonstrated the regulation in activity of membrane transporter systems by the major signaling pathways, i.e., PKA and PKC and Ca^{2+}/calmodulin-mediated pathways. We also investigated the possible regulation of the folic acid uptake process by intracellular regulatory pathways. Concentration dependent inhibition by TFP, KN-62 and calmidazolium suggested the involvement of Ca^{2+}/calmodulin mediated pathways in the regulation of folic acid uptake. PKA pathway modulators (IBMX, forskolin and H-89) and PKC pathway modulators (PMA and bisindolylmaleimide I) have shown significant inhibition in the uptake of folic acid which shows that these pathways play an important role in the regulation of uptake. The results from this study indicate the involvement of Ca^{2+}/calmodulin, PKA and PKC pathways in the regulation of folic acid uptake. Physiological mechanism(s) through which calmidazolium and PK pathways exert its regulatory effect on folic acid uptake is unclear. Most signal transduction pathways are involved in diverse and critical cellular functions. Extensive cross-signaling between cAMP and calmodulin-mediated signal transduction pathways exist at several levels of cellular control. Therefore, calmodulin mediated reduction of folic acid uptake in T47D cells might be a manifestation of the intertwined regulation of these processes. RT-PCR analysis provided evidence for molecular expression of FR-α at mRNA level. Quantitative real time PCR studies confirmed higher mRNA expression of FR-α in T47D cells (approximately 13 fold) compared with MCF-7 cells. Clear distinct bands in Western blot confirmed the presence of FR-α (37 kDa) protein in T47D cells. FR-α proteins are involved in the influx of folic acid into T47D BC cells. From the above discussion, the existence of a specific transport system for folic acid, FR-α, in T47D cells is evident.

Conclusion

This is the first report indicating the functional and molecular expression of FR-α in T47D cells. In future, folate carrier systems such as folate-conjugated nanoparticles and prodrugs can be designed and utilized for the targeted delivery drugs to BC cells. This approach enhances the permeability and improves the therapeutic outcomes of chemotherapeutic agents.

Acknowledgments

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