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Differential Expression of Folate Receptor-Alpha, Sodium-Dependent Multivitamin Transporter, and Amino Acid Transporter ($B^{(0,+)}$) in Human Retinoblastoma (Y-79) and Retinal Pigment Epithelial (ARPE-19) Cell Lines

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Abstract

Purpose: The overall objective of this study was to investigate the differential expression of folate receptor-alpha (FR-α), sodium-dependent multivitamin transporter (SMVT), and amino acid transporter [$B^{(0,+)}$] in retinoblastoma (Y-79) and retinal pigment epithelial (ARPE-19) cells.
Methods: Polymerase chain reaction (PCR) analysis was performed to confirm the existence of FR-α, SMVT, and $B^{(0,+)}$ in Y-79 and ARPE-19 cell lines. Quantitative real-time PCR was also performed to determine the relative expression of FR-α, SMVT, and $B^{(0,+)}$ at mRNA level in these cell lines. Quantitative uptake of [^{3}H] Folic acid, [^{3}H] Biotin, and [^{14}C] Arginine was studied in Y-79 and ARPE-19 cells. Further, saturation kinetics of [^{3}H] Folic acid, [^{3}H] Biotin, and [^{14}C] Arginine was performed in the presence of various concentrations of respective cold substrates to determine the kinetic parameters (K_m and V_{max}) in Y-79 and ARPE-19 cells.
Results: PCR analysis had confirmed the existence of FR-α, SMVT, and $B^{(0,+)}$ in Y-79 and ARPE-19 cells. Quantitative real-time PCR analysis had shown significantly higher expression of FR-α, SMVT, and $B^{(0,+)}$ mRNA levels in Y-79 cells compared with ARPE-19 cells. Quantitative uptake of [^{3}H] Folic acid, [^{3}H] Biotin, and [^{14}C] Arginine was found to be significantly higher in Y-79 cells relative to ARPE-19 cells. [^{3}H] Folic acid uptake process followed saturation kinetics with apparent K_m of 8.29 nM and V_{max} of 393.47 fmol/min/mg protein in Y-79 cells and K_m of 80.55 nM and V_{max} of 491.86 fmol/min/mg protein in ARPE-19 cells. [^{3}H] Biotin uptake process also displayed saturation kinetics with K_m of 8.53 μM and V_{max} of 14.12 pmol/min/mg protein in Y-79 cells and K_m of 138.25 μM and V_{max} of 38.85 pmol/min/mg protein in ARPE-19 cells. [^{14}C] Arginine uptake process followed saturation kinetics with K_m of 16.77 μM and V_{max} of 348.27 pmol/min/mg protein in Y-79 cells and K_m of 52.03 μM and V_{max} of 379.21 pmol/min/mg protein in ARPE-19 cells.
Conclusions: This work demonstrated for the first time the higher expression and affinity of FR-α, SMVT, and $B^{(0,+)}$ mRNA levels in retinoblastoma (Y-79) cells compared with retinal pigment epithelial (ARPE-19) cells.

Introduction

Retinoblastoma (RB) represents intraocular neoplasm particularly in children, with around 200 new cases being reported every year in the United States.[1,2] Approximately, 87% of children diagnosed with RB do not survive very long due to metastasis and hematogenous spread. RB is common in children between the age of 3 and 7.[3] Enucleation coupled with external beam radiotherapy (EBR) remains as the mainstay treatment for intraocular malignancies. Of late, there has been a resurgence of interest in implementing chemotherapy as a major treatment option in RB so as to avoid enucleation and/or EBR to save vision.[3] Moreover, chemotherapy also lowers the risk of secondary malignancies associated with EBR. Tumor regression is evident following a combination therapy with cyclophosphamide, doxorubicin, vincristine, thiotepa, nimustine, melphalan, and cisplatin.[4] However, for chemotherapy to be successful, the

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anticancer agents must generate intracellular therapeutic concentrations. Efflux pumps that are highly expressed on the cancer cell membrane are responsible for subtherapeutic levels of chemotherapeutic agents, leading to drug resistance. With multidrug resistance (MDR) gene overexpression, tumor cells may develop resistance to a wide range of structurally and functionally diverse chemical agents. MDR genes encode for 3 main types of efflux proteins: P-glycoprotein (P-gp), multidrug resistance associated proteins (MRP), and breast cancer resistance protein (BCRP).[5,6] Of these P-gp and MRPs are known to be expressed on RB cells. Most chemotherapeutic agents are good substrates for these efflux pumps that may reduce drug concentrations in tumor cells.[7,8]

Moreover, anticancer agents fail to differentiate between normal and tumor cells resulting in adverse effects and systemic toxicity. Over the past few years there has been a growing interest in the development of novel strategies for actively targeting drugs to cancer cells.[9] Nutrient transporters/receptors including amino acids, glucose, peptide, folate, biotin, riboflavin, monocarboxylic acid, nucleoside/nucleobase, organic anion/cation transporters, present on the retina, play an important role in nutrition and regulation of endogenous/exogenous substances.[10] Uncontrolled proliferation of cancer cells requires high intake of vitamins and nutrients as compared with normal cells. This requirement is often met by acquiring genetic mutations that functionally alter receptor-initiated signaling pathways in cancer cells. Such pathways in turn help in activating the uptake and metabolism of vitamins and nutrients necessary for promotion of cell survival and growth.[11,12] Cell membranes express a definite set of membrane transport proteins that bind to their respective substrates with high affinity and specificity.[13] Therefore novel approaches that can enhance the selectivity of anticancer agents to nutrient and vitamin transporters seem to be promising.

Transporter/receptor-mediated drug delivery is one such approach that has been successfully utilized for enhancing uptake across membranes.[14] By chemical modification or coupling to a promoeity (ligand) that is a substrate for the transporter/receptor, the parent drug can be transported.[15] Several transporter/receptor systems such as folate receptor-alpha (FR-α),[16] sodium-dependent multivitamin transporter (SMVT), and neutral and cationic amino acid transporter ($B^{(0,+)}$) play a critical role in the internalization of vitamins and amino acids. However, detailed understanding of the transporter/receptor overexpression in cancer cells can aid in designing an effective transporter/receptor-mediated drug delivery system. Therefore, our objective is to determine quantitative expression of FR-α, SMVT, and $B^{(0,+)}$ carrier–mediated systems on RB cells and compare with normal retinal cells. Y-79 and ARPE-19 cell lines were selected as models for RB and retinal pigment epithelial (RPE) cells, respectively. Various reports suggest that RB originates from a primordial bipotential neuroectodermal cell containing neuronal and glial characteristics. Hence Y-79 cell line maybe considered as a model of human retinal neoplasm.[2] Though several nutrient and vitamin transporters (peptide, amino acids, glucose, folate, monocarboxylic acid, nucleoside and nucleobase, organic anion, and organic cation transporters) are expressed on plasma membranes, this work investigates the differential expression of FR-α, SMVT, and $B^{(0,+)}$ carrier systems due to their importance in drug delivery.[17,18] Based on our studies and some published results from our laboratory we made an attempt to compile the differential expression of FR-α, SMVT, and $B^{(0,+)}$ carrier–mediated systems on RB cells and compare with ARPE-19 cells.

Methods

Materials

[^{3}H] Folic acid (50 Ci/mmol), [^{3}H] Biotin (50 Ci/mmol), and [^{14}C] Arginine (8.5 mCi/mmol) were procured from Perkin-Elmer (Boston, MA). Y-79 and ARPE-19 cells were obtained from ATCC (Manassas, VA). The growth medium and nonessential amino acids were obtained from Gibco (Invitrogen, GrandIsland, NY). Penicillin, streptomycin, sodium bicarbonate, and HEPES were purchased from Sigma-Aldrich (St. Louis, MO). Culture flasks (75 cm^2 growth area) and 12-well tissue culture plates were purchased from Corning Life Sciences (Lowell, MA). The buffer chemicals were of analytical grade and obtained from Sigma-Aldrich.

Cell culture

Y-79 cells were cultured as a suspension in RPMI 1640 medium supplemented with 15% non-heat-inactivated fetal bovine serum, 1 mM glutamine, penicillin (100 units/mL), and streptomycin (100 μg/mL). ARPE-19 cells (passages 18–25) were cultured in DMEM/F-12 containing 10% heat-inactivated fetal bovine serum, 15 mM HEPES, 29 mM sodium bicarbonate, penicillin (100 units/mL), and streptomycin (100 μg/mL). Cells were maintained in 75 cm^2 tissue culture flasks at 37°C, in a humidified atmosphere of 5% CO_2 and 90% relative humidity. The medium was replaced every other day. ARPE-19 cells were grown on 12-well costar plates. All the ARPE-19 cultures used for experimentation were multilayered and confluent.

Reverse transcription–polymerase chain reaction analysis

RNA was extracted from cells using TRIzol® reagent (Invitrogen, GrandIsland, NY) according to the manufacturer's protocol. Briefly, cells were lysed using phenol-chloroform and isopropanol method. RNA was suspended in DNase-RNase–free water and concentration was determined using Nanodrop (Thermo Scientific, Wilmington, DE). cDNA was generated for specific amount of mRNA using oligodT and M-MLV RT polymerase. Three microliters of cDNA was then introduced into polymerase chain reaction (PCR). Primers used for the amplification of specific genes were summarized in Table 1. The PCR conditions were as follows: denaturation (94°C, 45 s), annealing (56°C, 1 min), and extension (72°C, 45 s) for 45 amplification cycles, followed by a final extension of 72°C for 10 min. The product was then subjected to gel electrophoresis with 1.5% agarose gel and visualized under UV.

Quantitative real-time PCR analysis

Quantitative real-time PCR (qPCR) was performed to compare the FR-α, SMVT, and $B^{(0,+)}$ transporter expression levels in Y-79 and ARPE-19 cells. qPCR was performed with Light cycler SYBR-green technology (Roche) using cDNA equivalent to 80 ng of total RNA with specific primers for

Table 1. Polymerase Chain Reaction Primers for Folate Receptor-Alpha, Sodium-Dependent Vitamin Transporter, and Neutral and Cationic Amino Acid Transporter [B $^{(0,+)}$]

Gene		*Sequence 5′→3′*	*Product length*
FR-α	Forward	AGGACAGACATGGCTCAGCGGA	396
	Reverse	TACCCGCTCTTTGCGCCAGC	
SMVT	Forward	AGGGCTGCAGCGGTTCTATT	774
	Reverse	GCAGCTTCCAGTTTTATGGTGGAG	
B$^{(0,+)}$	Forward	CCAGCCGAGGGAGTGAACCATG	698
	Reverse	TTGACCGTTGGAGCGCCACTT	

FR-α, folate receptor-alpha; SMVT, sodium-dependent multivitamin transporter.

each gene. 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 mRNA in ARPE-19 with Y-79 cells. The real-time primers used for the study were summarized in Table 2. GAPDH was used as an internal control in both the cells.

Uptake experiments

Y-79 cells were collected following centrifugation and then washed 3 times with Dulbecco's phosphate-buffered saline (DPBS; pH 7.4), containing 130 mM NaCl, 0.03 mM KCl, 7.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, and 5 mM glucose. Aliquots of $\sim 5 \times 10^6$ cells were then preincubated in 1 mL DPBS for 10 min at 37°C. Uptake was initiated by the addition of a fixed amount of [^{3}H] Folic acid, [^{3}H] Biotin, and [^{14}C] Arginine. Cells were incubated for 30 min at 37°C. At the end of each experiment, tubes were immediately centrifuged, the solution was aspirated, and cells were washed 3× with 1 mL of ice-cold stop solution (210 mM KCl and 2 mM HEPES), pH 7.4, to arrest the reaction. Cells were then solubilized in 1 mL of 0.1% Triton-X solution in 1% NaOH and an aliquot was then transferred to vials containing 5 mL of scintillation cocktail. Radioactivity associated with the cells was quantified with a scintillation counter (Model LS-9000; Beckman Instruments, Inc., Fullerton, CA) and protein content of each sample was measured by the methods of Bradford with bovine serum albumin as the standard (Bio-Rad protein estimation kit; Hercules, CA). Cell viability under all treatment regimens was monitored by the trypan blue exclusion test and was routinely observed to be between 90% and 95%. Similarly, uptake studies were carried out with 21-day-old ARPE-19 cell monolayers. Following aspiration of the culture medium, cell monolayers were washed with DPBS thrice and uptake was performed as mentioned previously.[16]

Table 2. Quantitative Real-Time Polymerase Chain Reaction Primers for Folate Receptor-Alpha, Sodium-Dependent Vitamin Transporter, and Neutral and Cationic Amino Acid Transporter [B $^{(0,+)}$]

Gene		*Sequence 5′→3′*
FR-α	Forward	AGGACAGACATGGCTCAGCG
	Reverse	TGTGGTGCTTGGCGTTCATG
SMVT	Forward	TACCAGTTCTGCCAGCCACAGTG
	Reverse	CAGGGACACCAAAACCTCCCTCT
B$^{(0,+)}$	Forward	AGCCGAGGGAGTGAACCATG
	Reverse	GGACCAGTTACCACGGTCCT

Following the method just outlined, saturation kinetics of all the substrates was studied by performing the uptake in presence of various concentrations of unlabeled cold substrates.

Data analysis

Uptake data were fitted into a classical Michaelis–Menten equation.

$$V = \frac{Vmax * C}{Km + C}$$

V is the total rate of uptake, V_{max} represents the maximum uptake rate for the carrier-mediated process, K_m (Michaelis–Menten constant) is the concentration at half-saturation, and C denotes substrate concentration. Data were fitted to above equation with a nonlinear least square regression analysis program (Kaleida Graph Version 3.09; Synergy Software, PA) and the kinetic parameters were calculated. Quality of the fit was determined 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 6 times and results were expressed as mean ± SD. Unpaired Student's t-test was used to estimate statistical significance. A difference between mean values was considered significant if $P < 0.05$.

Results

Reverse transcription–PCR analysis

Expression of FR-α in ARPE-19 and Y-79 cells at the mRNA level was determined by reverse transcription–PCR (RT-PCR) analysis. A major band (396 bp) corresponding to the amplified FR-α precursor mRNA in Y-79 (Fig. 1A) and ARPE-19 cells (Fig. 1B) was noted by gel electrophoresis. BLAST analysis indicated that the primers used in this study could result in a PCR product of 396 bp by binding to the FR-α precursor mRNA. Similarly, the expression of SMVT in Y-79 and ARPE-19 cells at the mRNA level was also determined. A major band (774 bp) corresponding to the amplified SMVT precursor mRNA in Y-79 (Fig. 1A) and ARPE-19 cells (Fig. 1B) was observed in gel electrophoresis. BLAST analysis showed that the primers used in this study can result in a PCR product of 774 bp by binding to the SMVT precursor mRNA. The expression of the B $^{(0,+)}$ in Y-79 and ARPE-19 cells at the mRNA level was determined by RT-PCR analysis. A major band

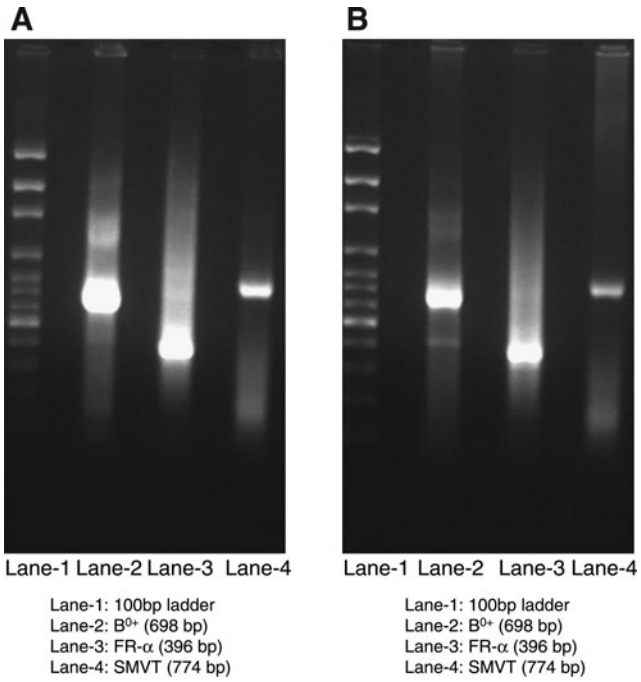

FIG. 1. **(A)** Molecular identity of B $^{(0,+)}$, FR-α, and SMVT in Y-79 cells at the mRNA level. PCR product obtained using B $^{(0,+)}$-, FR-α-, and SMVT-specific primers. Lane 1 represents 100-bp DNA ladder. Major bands were shown at ~698 (lane 2), ~396 (lane 3), and ~774 (lane 4) corresponding to the amplified B $^{(0,+)}$, FR-α, and SMVT cDNA. **(B)** Molecular identity of B $^{(0,+)}$, FR-α, and SMVT in ARPE-19 cells at the mRNA level. PCR product obtained using FR-α-, SMVT-, and B $^{(0,+)}$-specific primers. Lane 1 represents 100-bp DNA ladder. Major bands were shown at ~698 (lane 2), ~396 (lane 3), and ~774 (lane 4) corresponding to the amplified B $^{(0,+)}$, FR-α, and SMVT cDNA. FR-α, folate receptor-alpha; SMVT, sodium-dependent multivitamin transporter; PCR, polymerase chain reaction.

(698 bp) corresponding to the amplified B $^{(0,+)}$ precursor mRNA in Y-79 (Fig. 1A) and ARPE-19 cells (Fig. 1B) was observed in gel electrophoresis. BLAST analysis showed that the primers used in this study can result in a PCR product of 698 bp by binding to the B $^{(0,+)}$ precursor mRNA.

qPCR analysis

qPCR analysis was performed to compare the mRNA levels of FR-α, SMVT, and B $^{(0,+)}$ in Y-79 and ARPE-19 cells. FR-α expression at mRNA level was found to be 3 times higher in Y-79 than ARPE-19 cells. SMVT mRNA expression level was 6-fold higher in Y-79 relative to ARPE-19 cells. B $^{(0,+)}$ expression at mRNA level was found to be 17 times higher in Y-79 than ARPE-19 cells. These results clearly indicate the elevated expression of FR-α, SMVT, and B $^{(0,+)}$ in Y-79 relative to ARPE-19 cells (Fig. 2).

Quantitative uptake of [^{3}H] Folic acid, [^{3}H] Biotin, and [^{14}C] Arginine

Uptake of [^{3}H] Folic acid in Y-79 cells was found to be significantly higher compared to ARPE-19 cells. This could be due to high expression of FR-α in Y-79 cells than ARPE-19 cells Uptake of [^{3}H] Biotin in ARPE-19 cells was also found

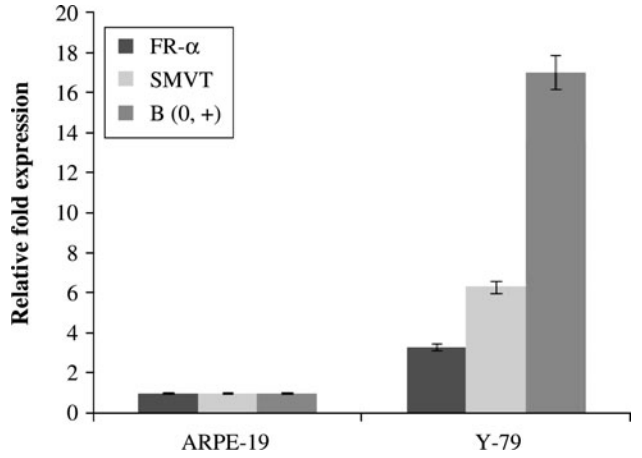

FIG. 2. Relative fold expression of FR-α, SMVT, and B $^{(0,+)}$ in ARPE-19 and Y-79 by quantitative real-time PCR analysis.

to be significantly lower relative to Y-79 cells. This result indicates robust expression of SMVT in Y-79 than ARPE-19 cells. Similarly uptake of [^{14}C] Arginine in Y-79 cells was found to be significantly higher relative to ARPE-19 cells. This observation indicates high expression of B $^{(0,+)}$ in Y-79 cells than ARPE-19 retinal cells (Fig. 3).

Concentration dependency of folic acid

Uptake kinetics of a carrier-mediated system for folic acid in Y-79 cells was determined earlier from our laboratory[19] by evaluating the saturation kinetics of [^{3}H] Folic acid in the presence of unlabeled folic acid. After fitting the data to Michaelis–Menten equation, an uptake process with apparent K_m of 0.0083 μM and V_{max} of 0.39 pmol/min/mg protein was obtained (Fig. 4A). Uptake kinetics of carrier-mediated system for folic acid in ARPE-19 cells was determined by evaluating the saturation kinetics of [^{3}H] Folic acid in the presence of unlabeled folic acid. After fitting the data to Michaelis–Menten equation, an uptake process with apparent K_m of 0.08 μM and V_{max} of 0.49 pmol/min/mg protein was observed (Fig. 4B).

Concentration dependency of biotin

Uptake kinetics of a carrier-mediated system for biotin in Y-79 cells was determined earlier from our laboratory[17] by

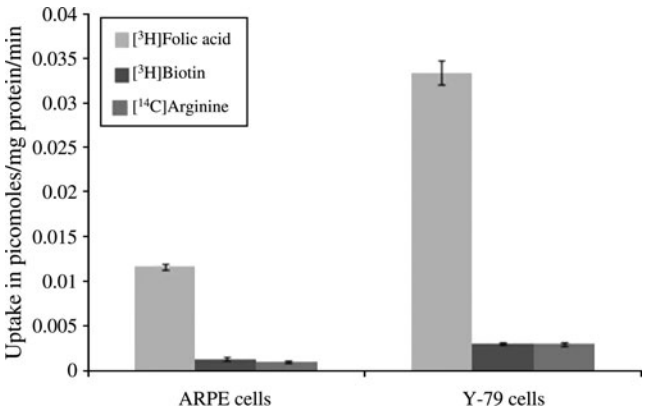

FIG. 3. Quantitative uptake of [^{3}H] Folic acid, [^{3}H] Biotin, and [^{14}C] Arginine in ARPE-19 and Y-79 cells.

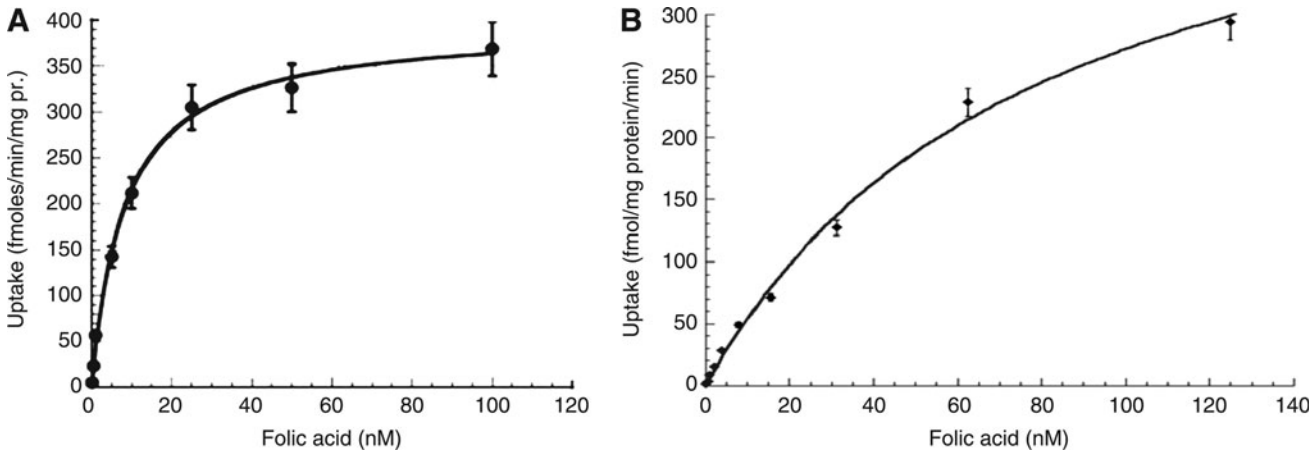

FIG. 4. **(A)** Uptake of [^{3}H] Folic acid by Y-79 cells as a function of substrate concentration at 37°C, pH 7.4. Reproduced with permission from Kansara et al.[19] **(B)** Uptake of [^{3}H] Folic acid by ARPE-19 cells as a function of substrate concentration at 37°C, pH 7.4. Each data point represents the mean ± SD of 4–6 separate uptake determinations.

evaluating saturation kinetics of [^{3}H] Biotin in the presence of unlabeled biotin. After fitting the data to Michaelis–Menten equation, an uptake process with apparent K_m of 8.53 μM and V_{max} of 14.12 pmol/min/mg protein was obtained (Fig. 5A). Uptake kinetics of a carrier-mediated system for biotin in ARPE-19 cells was determined earlier from our laboratory[20] by evaluating saturation kinetics of [^{3}H] Biotin in the presence of unlabeled biotin. After fitting the data to Michaelis–Menten equation, an uptake process with apparent K_m of 138.25 μM and V_{max} of 38.85 pmol/min/mg protein was obtained (Fig. 5B).

Concentration dependency of arginine

Uptake kinetics of a carrier-mediated system for arginine in the Y-79 cells was determined by evaluating the uptake kinetics of [^{14}C] Arginine in the presence of unlabeled arginine. After fitting the data to Michaelis–Menten equation, an uptake process with apparent K_m of 16.77 μM and V_{max} of 348.27 pmol/min/mg protein was obtained (Fig. 6A). Uptake kinetics of a carrier-mediated system for arginine in the ARPE-19 cells was determined by evaluating the saturation kinetics of [^{14}C] Arginine in the presence of unlabeled arginine. After fitting the data to Michaelis–Menten equation, an uptake process with apparent K_m of 52.03 μM and V_{max} of 379.21 pmol/min/mg protein was observed (Fig. 6B).

Discussion

Selective targeting reduces the minimum effective dose as well as associated toxicity and also enhances the therapeutic efficacy with equivalent plasma concentrations.[21,22] Overexpression of vitamin and amino acid transporters/receptors, by a variety of tumor cell lines, may present an opportunity for targeted delivery of anticancer drugs for the treatment of various malignancies and diagnostic imaging. Most chemotherapeutic agents fail to enter tumor cells in therapeutic concentrations due to presence of efflux pumps (P-glycoprotein, MDR-associated protein, lung resistance-related protein, and BCRP).[3] Vitamins and amino acids as

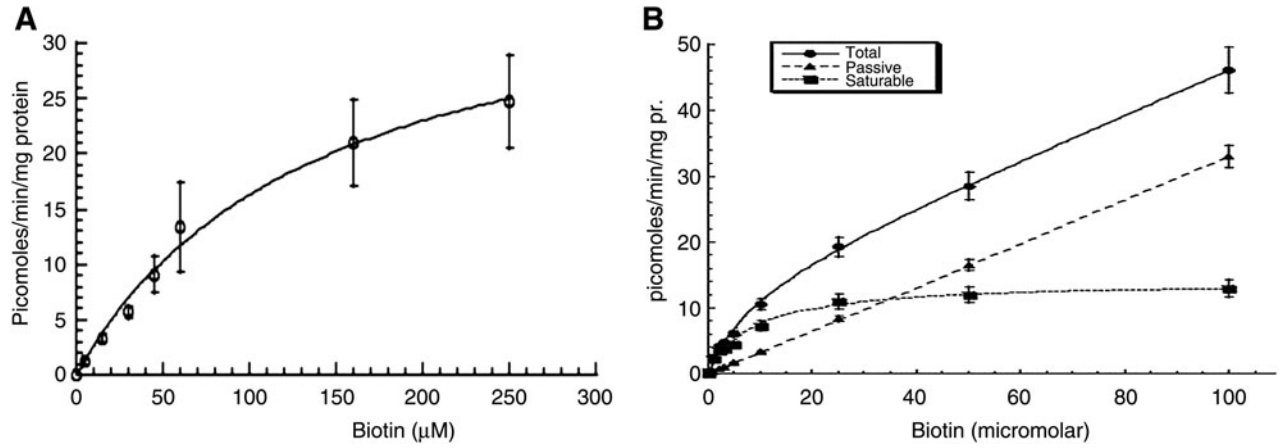

FIG. 5. **(A)** Uptake of [^{3}H] Biotin by Y-79 cells as a function of substrate concentration at 37°C, pH 7.4. Each data point represents the mean ± SD of 4–6 separate uptake determinations. Modified with permission from Kansara et al.[17] **(B)** Uptake of [^{3}H] Biotin by ARPE-19 cells as a function of substrate concentration at 37°C, pH 7.4. Each data point represents the mean ± SD of 4–6 separate uptake determinations. Reproduced with permission from Janoria et al.[20]

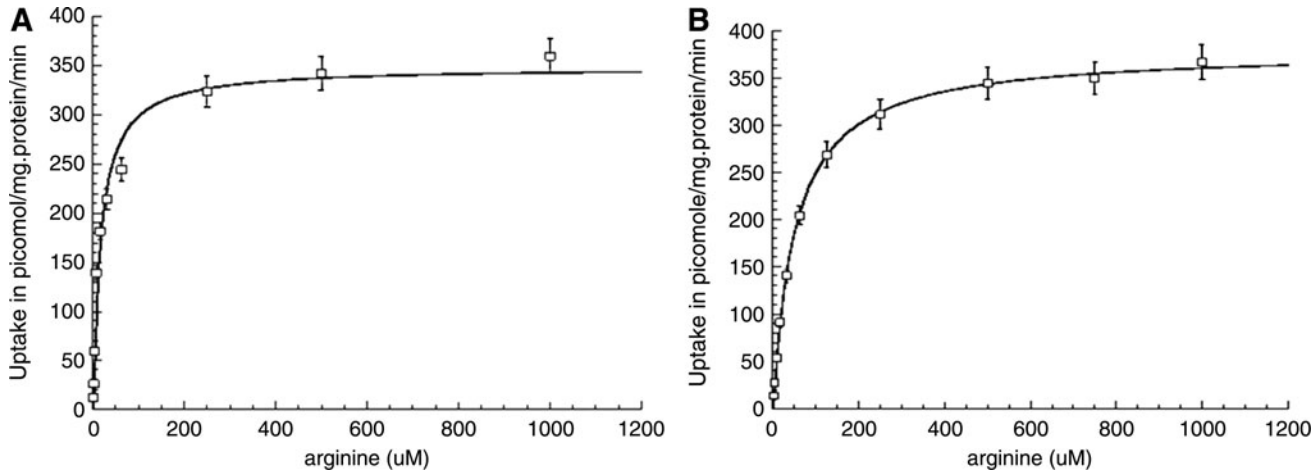

FIG. 6. **(A)** Uptake of [^{14}C] Arginine by Y-79 cells as a function of substrate concentration at 37°C, pH 7.4. **(B)** Uptake of [^{14}C] Arginine by ARPE-19 cells as a function of substrate concentration at 37°C, pH 7.4. Each data point represents the mean ± SD of 4–6 separate uptake determinations.

targeting moieties offer prospective advantages than macromolecules such as antibodies. These include the following: (a) small molecular size of the targeting moiety offers favorable pharmacokinetic properties and decreased probability of immunological reactions, which allows chronic administration; (b) low cost and availability; (c) high affinity for receptors; (d) ligand can be internalized into tumor cells by endocytosis, which aids in cytosolic delivery of therapeutic agents; and (e) high frequency of expression among cancer cell lines with potential for future therapeutic and diagnostic applications.[23]

Human retina is a delicate organization of neurons, glia, and nourishing blood vessels. Biotin, an essential water-soluble vitamin, appears to play a critical role in cellular homeostasis and pathological conditions of retina including RB. SMVT expressed on the retina maybe targeted following systemic and intravitreal administration to generate enhanced drug availability in the RPE, choroids, and Bruch's membrane. Amino acid transporters are widely expressed and contribute to uptake of neurotransmitters and nutrients.[24] B $^{(0,+)}$ is a sodium-dependent neutral and cationic amino acid transporter. It transports over 20 proteinogenic amino acids. This transporter has the ability to translocate amino acids in their L-isomeric form and some in their D-isomeric form. The protein acts in an energy-dependent manner. This requires a combination of Na^+ and Cl^- transmembrane gradient and membrane potential. The transporter can concentrate substrates 1000-fold more inside cells.[25] In addition to its amino acid transport, it can also transport carnitine, D-serine, NOS (nitric oxide synthase) inhibitors, conjugated drugs, and prodrugs. This property makes it unique among amino acid transporters.[26] Furthermore, structural requirements for translocation are not very stringent. An absence of negative charge on the side chain of the amino acid is the only structural requirement. It is highly expressed in lung, colon, and ocular tissues.[27,28] These factors together offer a broad choice for the design of prodrugs.

The aim of this study was to evaluate the expression of FR-α, SMVT, and B $^{(0,+)}$ carrier systems in Y-79 cells relative to ARPE-19 cells. Y-79 cell line was selected as a model system as it is derived from a tumor of the inner plexiform layer of the retina and has several membrane properties of the retina.[29] ARPE-19 cell line is yet another widely used human origin retinal cell line. ARPE-19 cells are similar to RPE in terms of epithelial morphology, polarization, and expression of various ion channels, transporters, and RPE-specific markers.[20,30,31] RT-PCR analysis provided the preliminary evidence of the molecular expression of FR-α, SMVT, and B $^{(0,+)}$ carrier systems in ARPE-19 and Y-79 cells. This was further confirmed by qPCR that allows the quantification of gene expression. Quantitative uptake of [^{3}H] Folic acid, [^{3}H] Biotin, and [^{14}C] Arginine was significantly higher in Y-79 cells as compared with ARPE-19 cells. This could be attributed to the higher expression of FR-α, SMVT, and B $^{(0,+)}$ carrier systems in Y-79 cells than that of ARPE-19 cells. Tumor cells require a constant supply of nutrients for their uninterrupted growth and in order to facilitate the transport of nutrients, they express higher levels of nutrient transporters.[32] Saturation kinetics of [^{3}H] Folic acid, [^{3}H] Biotin, and [^{14}C] Arginine was studied by performing the uptake in presence of various concentrations of unlabeled cold substrates. [^{3}H] Folic acid uptake process followed saturation kinetics with apparent K_m of 8.29 nM and V_{max} of 393.47 fmol/min/mg protein in Y-79 cells and K_m of 80.55 nM and V_{max} of 491.86 fmol/min/mg protein in ARPE-19 cells. [^{3}H] Biotin uptake process also displayed saturation kinetics with K_m of 8.53 μM and V_{max} of 14.12 pmol/min/mg protein in Y-79 cells and K_m of 138.25 μM and V_{max} of 38.85 pmol/min/mg protein in ARPE-19 cells. [^{14}C] Arginine uptake process followed saturation kinetics with K_m of 16.77 μM and V_{max} of 348.27 pmol/min/mg protein in Y-79 cells and K_m of 52.03 μM and V_{max} of 379.21 pmol/min/mg protein in ARPE-19 cells. These results provide additional support for the presence and higher expression of FR-α, SMVT, and B $^{(0,+)}$ carrier systems in Y-79 cells. V_{max} and K_m are the 2 important parameters that define the functional and kinetic behavior of a transporter protein as a function of substrate concentrations. K_m value represents the strength of binding and affinity of substrates. K_m value depends on several factors including substrate concentration, temperature, and pH. K_m is a measure of apparent binding affinity of the respective substrate toward the transporter. Low K_m

value indicates a tightly bound substrate, while loosely bound substrates have high K_m values. V_{max} depends on the structure and concentration of the transporter protein and is a measure of the drug translocation capacity of the carrier system. Based on the saturation kinetics, the K_m values of [^{3}H] Folic acid, [^{3}H] Biotin, and [^{14}C] Arginine were found to be relatively lower in Y-79 cells, indicating the higher binding strength and affinity of substrates in cancerous cells. All of the results tend to suggest that FR-α, SMVT, and B $^{(0,+)}$ are overexpressed in Y-79 than ARPE-19 cells. Enhanced expression of such transporters and receptors on Y-79 cells may provide new opportunities for transporter-targeted prodrug design for enhanced drug delivery to treat RB.

Conclusion

In conclusion, this is the first study showing the differential expression of specialized FR-α, SMVT, and B $^{(0,+)}$ carrier systems in Y-79 relative to ARPE-19 cells. Higher expression levels of FR-α, SMVT, and B$^{(0,+)}$ carrier systems on RB cells may be suitable for the design of surface-modified nanoparticles and transporter-targeted prodrugs to achieve enhanced permeability of highly potent, but poorly bioavailable, drugs. A small increase in ocular bioavailability could significantly increase the therapeutic response of drugs.

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Author Disclosure Statement

No competing financial interests exist.

References

1. Parkin, D.M., Stiller, C.A., Draper, G.J., and Bieber, C.A. The international incidence of childhood cancer. *Int. J. Cancer* 42:511–520, 1988.
2. Kyritsis, A.P., Tsokos, M., Triche, T.J., and Chader, G.J. Retinoblastoma—origin from a primitive neuroectodermal cell? *Nature* 307:471–473, 1984.
3. Boddu, S.H., Jwala, J., Chowdhury, M.R., and Mitra, A.K. *In vitro* evaluation of a targeted and sustained release system for retinoblastoma cells using Doxorubicin as a model drug. *J. Ocul. Pharmacol. Ther*. 26:459–468, 2010.
4. Chan, H.S., Gallie, B.L., Munier, F.L., and Beck Popovic, M. Chemotherapy for retinoblastoma. *Ophthalmol. Clin. North Am*. 18:55–63, viii, 2005.
5. Amaral, L., Engi, H., Viveiros, M., and Molnar, J. Review. Comparison of multidrug resistant efflux pumps of cancer and bacterial cells with respect to the same inhibitory agents. *In Vivo* 21:237–244, 2007.
6. Yamamoto, Y., Shimizu, E., Masuda, N., Takada, M., and Sone, S. RB protein status and chemosensitivity in non-small cell lung cancers. *Oncol. Rep*. 5:447–451, 1998.
7. Kartner, N., Evernden-Porelle, D., Bradley, G., and Ling, V. Detection of P-glycoprotein in multidrug-resistant cell lines by monoclonal antibodies. *Nature* 316:820–823, 1985.
8. Li, B., Gao, R., Zhang, H., Li, L.Q., Gao, F., and Ren, R.J. [Studies on multidrug resistance associated protein in retinoblastoma]. *Zhonghua Yan Ke Za Zhi*. 45:314–317, 2009.
9. Dean, M., Fojo, T., and Bates, S. Tumour stem cells and drug resistance. *Nat. Rev. Cancer* 5:275–284, 2005.
10. Kansara, V., Pal, D., Jain, R., and Mitra, A.K. Identification and functional characterization of riboflavin transporter in human-derived retinoblastoma cell line (Y-79): mechanisms of cellular uptake and translocation. *J. Ocul. Pharmacol. Ther*. 21:275–287, 2005.
11. Hsu, P.P., and Sabatini, D.M. Cancer cell metabolism: warburg and beyond. *Cell* 134:703–707, 2008.
12. Vander Heiden, M.G., Cantley, L.C., and Thompson, C.B. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 324:1029–1033, 2009.
13. Brzezinska, A., Winska, P., and Balinska, M. Cellular aspects of folate and antifolate membrane transport. *Acta. Biochim. Pol*. 47:735–749, 2000.
14. Dey, S., and Mitra, A.K. Transporters and receptors in ocular drug delivery: opportunities and challenges. *Expert Opin. Drug. Deliv*. 2:201–204, 2005.
15. Yang, C., Tirucherai, G.S., and Mitra, A.K. Prodrug based optimal drug delivery via membrane transporter/receptor. *Expert Opin. Biol. Ther*. 1:159–175, 2001.
16. Jwala, J., Boddu, S.H., Paturi, D.K., Shah, S., Smith, S.B., Pal, D., and Mitra, A.K. Functional characterization of folate transport proteins in Staten's Seruminstitut rabbit corneal epithelial cell line. *Curr. Eye. Res*. 36:404–416, 2011.
17. Kansara, V., Luo, S., Balasubrahmanyam, B., Pal, D., and Mitra, A.K. Biotin uptake and cellular translocation in human derived retinoblastoma cell line (Y-79): a role of hSMVT system. *Int. J. Pharm*. 312:43–52, 2006.
18. Gaudana, R., Jwala, J., Boddu, S.H., and Mitra, A.K. Recent perspectives in ocular drug delivery. *Pharm. Res*. 26:1197–1216, 2009.
19. Kansara, V., Paturi, D., Luo, S., Gaudana, R., and Mitra, A.K. Folic acid transport via high affinity carrier-mediated system in human retinoblastoma cells. *Int. J. Pharm*. 355:210–219, 2008.
20. Janoria, K.G., Boddu, S.H., Wang, Z., Paturi, D.K., Samanta, S., Pal, D., and Mitra, A.K. Vitreal pharmacokinetics of biotinylated ganciclovir: role of sodium-dependent multivitamin transporter expressed on retina. *J. Ocul. Pharmacol. Ther*. 25:39–49, 2009.
21. Garcia-Bennett, A., Nees, M., and Fadeel, B. In search of the Holy Grail: folate-targeted nanoparticles for cancer therapy. *Biochem. Pharmacol*. 81:976–984, 2011.
22. Pan, X., and Lee, R.J. Tumour-selective drug delivery via folate receptor-targeted liposomes. *Expert Opin. Drug. Deliv*. 1:7–17, 2004.
23. Sudimack, J., and Lee, R.J. Targeted drug delivery via the folate receptor. *Adv. drug deliv. rev.* 41:147–162, 2000.
24. Uchiyama, T., Fujita, T., Gukasyan, H.J., Kim, K.J., Borok, Z., Crandall, E.D., and Lee, V.H. Functional characterization and cloning of amino acid transporter B(0,+) (ATB(0,+)) in primary cultured rat pneumocytes. *J. Cell Physiol*. 214:645–654, 2008.
25. Karunakaran, S., Umapathy, N.S., Thangaraju, M., Hatanaka, T., Itagaki, S., Munn, D.H., Prasad, P.D., and Ganapathy, V. Interaction of tryptophan derivatives with SLC6A14 (ATB0,+) reveals the potential of the transporter as a drug target for cancer chemotherapy. *Biochem. J*. 414:343–355, 2008.
26. Ganapathy, M.E., and Ganapathy, V. Amino Acid Transporter ATB0,+ as a delivery system for drugs and prodrugs. *Curr. Drug Targets Immune Endocr. Metabol. Disord*. 5:357–364, 2005.
27. Jain-Vakkalagadda, B., Pal, D., Gunda, S., Nashed, Y., Ganapathy, V., and Mitra, A.K. Identification of a Na+-dependent cationic and neutral amino acid transporter,

B(0,+), in human and rabbit cornea. *Mol. Pharm*. 1:338–346, 2004.
28. Hatanaka, T., Haramura, M., Fei, Y.J., Miyauchi, S., Bridges, C.C., Ganapathy, P.S., Smith, S.B., Ganapathy, V., and Ganapathy, M.E. Transport of amino acid-based prodrugs by the Na+- and Cl(-) -coupled amino acid transporter ATB0,+ and expression of the transporter in tissues amenable for drug delivery. *J. Pharmacol. Exp. Ther*. 308:1138–1147, 2004.
29. Tombran-Tink, J., Li, A., Johnson, M.A., Johnson, L.V., Chader, G.J. Neurotrophic activity of interphotoreceptor matrix on human Y79 retinoblastoma cells. *J. Comp. Neurol*. 317:175–186, 1992.
30. Ablonczy, Z., Dahrouj, M., Tang, P.H., Liu, Y., Sambamurti, K., Marmorstein, A.D., and Crosson, C.E. Human Retinal Pigment Epithelium Cells as Functional Models for the RPE In Vivo. *Invest. Ophthalmol. Vis. Sci*. 52:8614–8620, 2011.
31. Dunn, K.C., Aotaki-Keen, A.E., Putkey, F.R., and Hjelmeland, L.M. ARPE-19, a human retinal pigment epithelial cell line with differentiated properties. *Exp. Eye Res*. 62:155–169, 1996.
32. Fuchs, B.C., and Bode, B.P. Amino acid transporters ASCT2 and LAT1 in cancer: partners in crime? *Semin. Cancer Biol*. 15:254–266, 2005.

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