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Functional Characterization of Sodium-Dependent Multivitamin Transporter in MDCK-MDR1 Cells and Its Utilization as a Target for Drug Delivery

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Abstract: The objective of this research is to characterize a sodium-dependent multivitamin transporter (SMVT) in MDCK-MDR1 cells (Madin-Darby canine kidney cells transfected with the human MDR1 gene) and to investigate the feasibility of utilizing the MDCK-MDR1 cell line as an in vitro model to study the permeability of biotin-conjugated prodrugs of anti-HIV protease inhibitors. Mechanism of [^{3}H]biotin uptake and transport was delineated. Transepithelial permeability of the biotin-conjugated prodrug, i.e., biotin-saquinavir, was also studied. Reverse transcription polymerase chain reaction (RT-PCR) was carried out to confirm the existence of SMVT in MDCK-MDR1 cells. Biotin uptake was Na^+, pH, and temperature dependent, but energy independent. Uptake of biotin was found to be saturable with a K_m of 13.0 μM, V_{max} 21.5 of pmol min^{-1} (mg of protein)$^{-1}$, and K_d of 0.12 μL min^{-1} (mg of protein)$^{-1}$. Both apical and basal uptake and transepithelial transport of [^{3}H]biotin showed that SMVT localized predominantly on the apical membrane of MDCK-MDR1 cells. [^{3}H]Biotin uptake was inhibited by excess unlabeled biotin and its structural analogues, i.e., desthiolbiotin and valeric acid, and other vitamins such as lipoic acid and pantothenic acid, but not by acetic acid, benzoic acid, biotin methyl ester, and biocytin. Biotin-saquinavir caused lowering of [^{3}H]biotin uptake, which indicates that it is recognized by SMVT. Apical to basal transport of [^{3}H]biotin was also significantly inhibited in the presence of excess biotin or biotin-saquinavir. Transepithelial transport studies of biotin-saquinavir in MDCK-MDR1, wild type MDCK, and Caco-2 cells revealed that permeability of biotin-saquinavir was similar in all three cell lines. A band of SMVT mRNA at 862 bp was identified by RT-PCR. A sodium-dependent multivitamin transporter, SMVT, responsible for biotin uptake and transport, was identified and functionally characterized in MDCK-MDR1 cells. Therefore, the MDCK-MDR1 cell line may be utilized as an in vitro model to study the permeability of biotin-conjugated prodrugs such as HIV protease inhibitors.

Keywords: Sodium-dependent multivitamin transporter (SMVT); MDCK-MDR1; biotin-saquinavir

Introduction

Biotin is a water soluble vitamin essential for normal cellular growth, function, and development. It functions as a cofactor for the carboxylases that catalyze a variety of metabolic reactions including fatty acid biosynthesis, gluconeogenesis, and catabolism of several branched chain amino acids and odd-chain fatty acids.[1−3] Severe biotin deficiency leads to a wide range of clinical abnormalities,

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such as neurologic disorders, growth retardation, and skin abnormalities.[1−4] Biotin can only be synthesized by bacteria, yeasts, molds, algae, and some plant species. Human and other mammals cannot synthesize it, therefore must obtain from exogenous sources. The major tissues for biotin absorption and metabolism are intestine,[7−13,18−20] placenta,[5,17−20] and liver.[8,11,18,20] Kidney[6,9,10,18−20] is also involved in biotin reabsorption and elimination. Mechanism of biotin uptake has recently been studied in various mammalian cells from human, rat, and rabbit.[5−20] Grassel et al.[5] were the first to report the presence of a sodium-dependent multivitamin transporter (SMVT) in human placenta that is responsible for the uptake of biotin, pantothenate, and lipoate. Later, a second biotin-specific high affinity system was reported in human keratinocytes and human peripheral blood mononuclear cells (PBMC).[14−15] Recently, Said and his group[7−13] studied the mechanism of biotin uptake in rat and human intestinal, liver, and renal epithelial cells and suggested that SMVT is the primary transport system responsible for biotin uptake. SMVT has been also identified by several other groups as the major carrier for biotin, pantothenate, and lipoate in several mammal cells.[5,14−20] Cloning and functional expression of a SMVT for biotin, lipoic acid, and pantothenic acid from intestinal and placental tissues have been reported.[17−20] Structural and functional analyses of SMVT suggest that a single transport system probably mediates uptake of biotin, pantothenate, and lipoate in the major absorptive tissues.[18]

Recently, considerable attention has been paid to target this nutrient transport system (SMVT) for delivering drugs with poor permeability.[21−24] For example, the biotin modified R.I.-K(biotin)-Tat9 exhibited significantly improved permeability over the parent drug R.I.-K-Tat9 probably due to carrier-mediated uptake by SMVT.[21] A conjugate of camptothecin-poly(ethylene glycol) (CPT-PEG) with biotin enhanced the delivery of parent drug CPT. Similarly, biotin-conjugated saquinavir-poly(ethylene glycol) enhanced oral bioavailability of saquinavir.[21−23]

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In this study, we investigated biotin uptake and transport in MDCK-MDR1 cells. This cell line was derived from Madin-Darby canine kidney cells which were transfected with the human MDR1 gene. We selected this cell line because it has been widely employed as an alternative to the Caco-2 model for high throughput drug permeability screening.[26−28] MDCK-MDR1 cells express a high level of P-gp and differentiate rapidly, and this model is a good in vitro model for studying the permeability of P-gp substrates and P-gp-mediated drug−drug interactions.[26−28] Several other transporters such as monocarboxylic acid (MCT) transporter, large neutral amino acid, bile acid, and peptide transporters expressed in MDCK and Caco-2 cells have been preliminarily screened.[25] However, no previous work about the SMVT transport system in the MDCK-MDR1 cell line has been reported. Hence, in this article, we report functional and molecular characterizations of SMVT in MDCK-MDR1 cells. The aim of this work is to investigate the feasibility of selecting the MDCK-MDR1 cell line as an in vitro model to study the permeability of biotin-conjugated anti-HIV protease inhibitors, e.g., saquinavir.

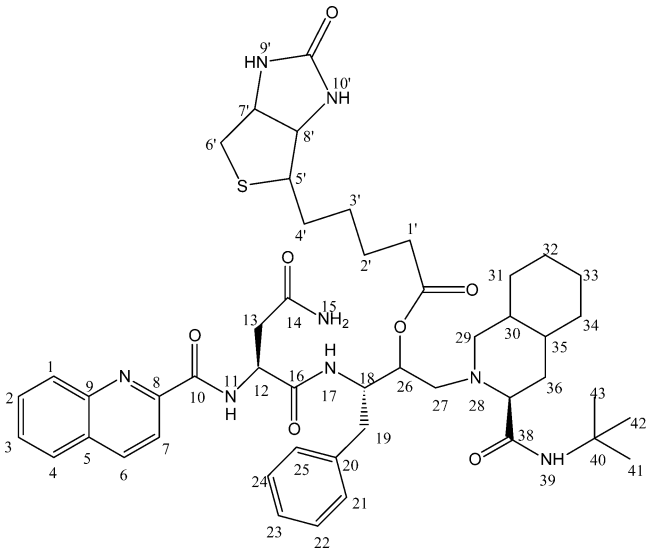
Materials and Methods

Materials. D-[8,9-^{3}H]Biotin (specific activity 50 Ci/mmol, radiochemical purity 97%) was purchased from Perkin-Elmer Life Science, Inc. (Boston, MA). D-Biotin was obtained from Fisher Scientific Co. (Fair Lawn, NJ). Pantothenic acid, lipoic acid, desthiolbiotin, biotin methyl ester, biocytin, acetic acid, benzoic acid, and valeric acid were procured from Sigma Chemical Company (St. Louis, MO). Saquinavir as its mesylate salt was kindly donated by Hoffmann-La Roche.

MDCK-MDR1 cells were donated by P. Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands). Dulbecco modified Eagle medium (DMEM), nonessential amino acids, calf serum (CS), and trypsin/EDTA were obtained from Gibco (Invitrogen, Grand Island, NY). Penicillin, streptomycin, sodium bicarbonate, and HEPES were purchased from Sigma Chemical Company (St. Louis, MO).

Dulbecco modified phosphate buffer saline (DPBS) was prepared with 129 mM NaCl, 2.5 mM KCl, 7.4 mM Na_2HPO_4, 1.3 mM KH_2PO_4, 1 mM $CaCl_2$, 0.7 mM $MgSO_4$, and 5.3 mM glucose at pH 7.4. DPBS also contained 20 mM HEPES. These chemicals were of analytical grade and obtained from Sigma. Culture flasks (75 cm^2 growth area), polyester Transwells (pore size of 0.4 μm and 12 mm diameter), and 12-well tissue culture treated plastic plates were purchased from Costar (Cambridge, MA). Buffer components and other solvents were obtained from Fisher Scientific Co. (Fair Lawn, NJ).

Chart 1. Structure of Biotin-Saquinavir



Methods. Synthesis of Biotin-Conjugated Saquinavir (Biotin-Saquinavir). Biotin (0.13 g, 0.52 mmol), saquinavir (0.2 g, 0.26 mmol), dicyclohexylcarbodiimide (DCC, 0.11 g, 0.52 mmol), and 4-(*N*,*N*-dimethylamino)pyridine (DMAP, 0.064 g, 0.52 mmol) were dissolved in dry DMF (15 mL) under a nitrogen atmosphere. The mixture was stirred continuously for 1 h at 0 °C and then allowed to warm up to room temperature. After 24 h, the reaction was checked by TLC (1:8 methanol/dichloromethane) and LC/MS analysis and was found to be complete. The urea derivative was removed by filtration, and the filtrate was washed with ethyl acetate. Solvents from combined filtrate and washing were totally removed under reduced pressure. The oily crude was washed with ether, solvent was evaporated, and the product was purified by silica gel chromatography (1:8 methanol/dichloromethane). Biotin-saquinavir is a white amorphous solid. It was kept under vacuum overnight to yield a dry product. The yield was 82%. The structure and purity were confirmed by TLC, LC/MS, LC/MS/MS, and NMR. The structure of biotin-saquinavir is shown in Chart 1. The MS spectrum along with the assignment of its ion fragments is shown in Scheme 1.

ESI-MS (MH^+): 897.5. Calcd ($C_{48}H_{46}N_8O_7S$): 896.5.

^{1}H NMR (DMSO-d_6): δ 8.84 (1H, d, H^{11}), 8.60 (1H, d, H^7), 8.23 (1H, bd, H^{39}), 8.18 (2H, d, $H^{1,6}$), 8.12 (1H, m, H^4), 7.89 (1H, m, H^2), 7.75 (1H, m, H^3), 7.48 (2H, bd, $H^{15,17}$), 6.98−7.17 (5H, m, H^{21-25}), 5.31 (1H, m, H^{26}), 4.76 (1H, m, H^{12}), 4.36 (1H, m, H^{18}), 3.29 (1H, m, H^{29}), 2.29−2.89 (7H, m, $H^{13,19,27,37}$), 1.32 (9H, s, H^{41-43}), 1.34−1.93 (13H, m,

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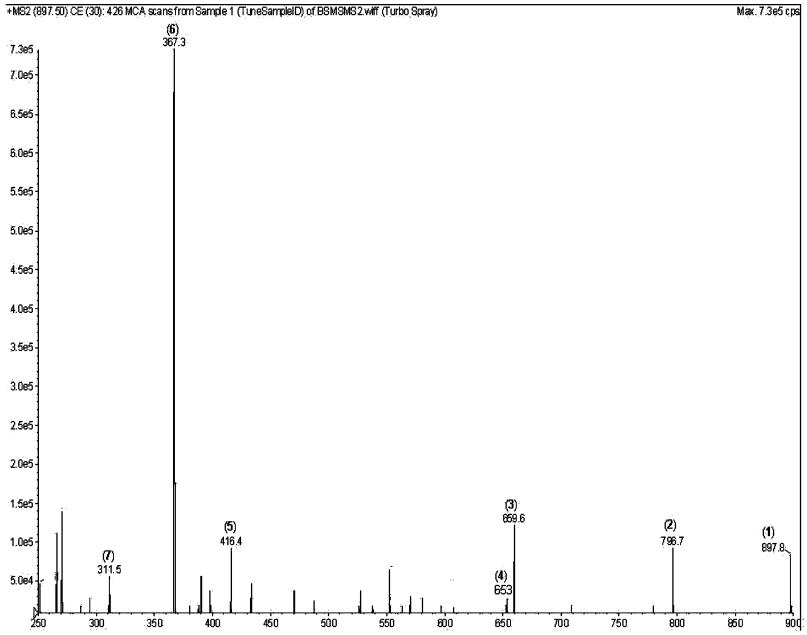
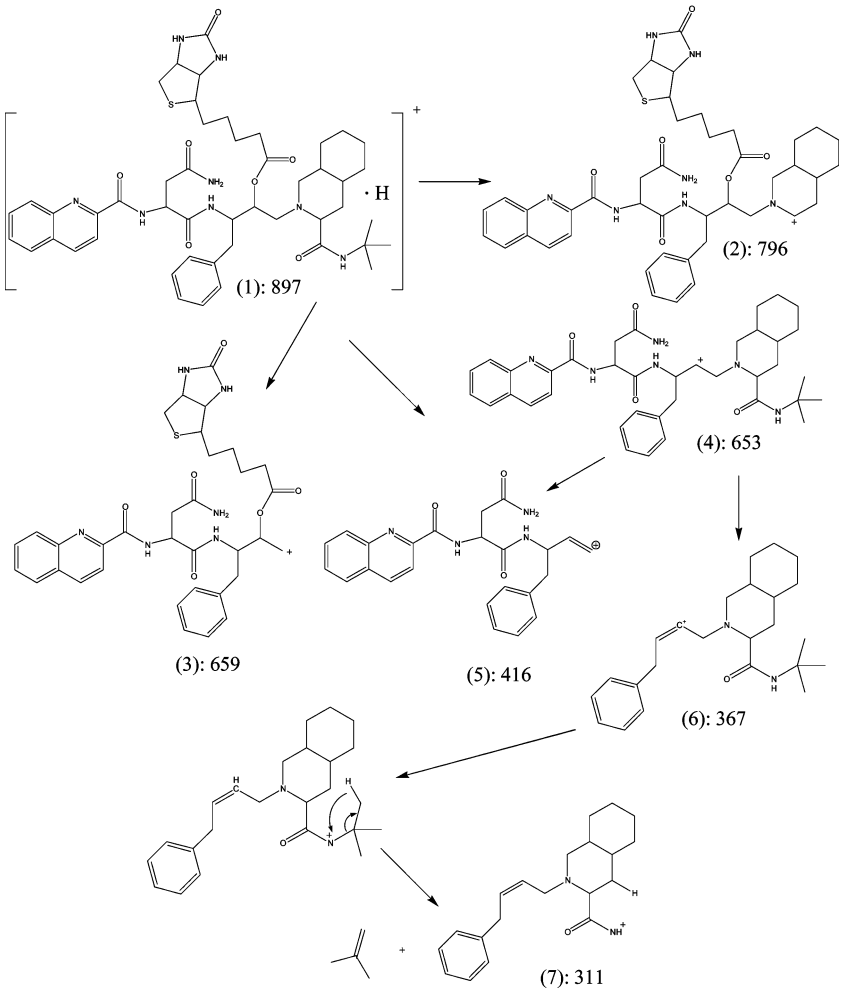
Scheme 1 . MS Spectrum and Assignment of Ion Fragments of Biotin-Saquinavir





H^{30-36}), 6.43 (1H, s, $H^{9'}$), 6.38 (1H, s, $H^{10'}$), 4.30 (1H, m, $H^{7'}$), 4.13 38 (1H, m, $H^{8'}$), 3.09 (1H, m, $H^{5'}$), 2.80 (2H, s, $H^{6'}$), 2.56 (2H, m, $H^{1'}$), 2.29 (2H, m, $H^{2'}$), 1.34−1.93 (4H, m, $H^{3',4'}$).

Cell Culture. MDCK-MDR1 cells (passages 5−15) were cultured in DMEM supplemented with 10% CS (heat inactivated), 1% nonessential amino acids, 100 units/mL penicillin, 100 g/mL streptomycin, 20 mM HEPES, and 29 mM sodium bicarbonate at pH 7.4. Cells were allowed to grow at 37 °C in a tissue culture incubator with 5% CO_2 and 95% air for 3−4 days to reach 80% confluence, and then were plated at a density of 100 000/cm^2 in 12-well tissue culture treated plastic plates. Cells were then incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air and grown for 6−7 days to reach confluence. The medium was changed every other day.

Uptake Experiments. Cell monolayers were rinsed 3 times, 10 min each with 2 mL of DPBS buffer at 37 °C, unless otherwise stated. Then, 1 mL of biotin solution ([^{3}H]-biotin, 0.5 μCi/mL) in DPBS buffer was added to each well and incubated at 37 °C for 5 min, unless otherwise mentioned (see Results). One-half milliliter and 1.5 mL of [^{3}H]biotin solution were added to the apical and basal chambers of a 12-well Transwell plate for apical and basal uptake, respectively. After the incubation period, the cell monolayers were rinsed three times with ice-cold stop solution (200 mM KCl and 2 mM HEPES) to terminate drug 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). 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 the BioRad protein estimation kit (BioRad, Hercules, CA), and the rate of uptake was normalized to the protein content of each well.

Na^+ Dependence. To study Na^+ dependence, sodium chloride and sodium phosphate in the incubation media were replaced by equimolar choline chloride and potassium phosphate, to generate sodium free medium. Cells were washed with either regular DPBS or sodium free medium. Solutions ([^{3}H]biotin, 0.5 μCi/mL) were prepared in either regular DPBS or sodium free media.

pH Dependence. DPBS incubation media were adjusted at different pH values (5.5, 6.0, 6.5, 7.0, 7.4, 8.0) by adding hydrochloric acid or sodium hydroxide. Cells were washed with DPBS of different pH values. Permeant solutions ([^{3}H]-biotin, 0.5 μCi/mL) were prepared in DPBS having different pH values.

Energy Dependence. After being washed with DPBS buffer 3 × 10 min, cells were preincubated with 1 mM metabolic inhibitors, i.e., 2,4-dinitrophenol, ouabain, and sodium azide at 37 °C for 30 min. Then an uptake study was conducted according to the procedure described previously.

Saturation Kinetics of Biotin Uptake (Concentration Dependence). Unlabeled biotin was first dissolved in DMSO to make stock solution, and then various concentrations (1−100 μM) of solutions were prepared in DPBS containing DMSO (≤1%). [^{3}H]Biotin was added to each tube containing different concentrations of unlabeled biotin to make donor solutions. Then, concentration-dependent uptake of biotin was carried out. The data was fitted to a modified Michaelis−Menten equation, and the apparent affinity constant (K_m), maximum uptake velocity (V_{max}), and passive diffusion constant (K_d) of Na^+-dependent biotin uptake were determined.

Inhibition Study (Substrate Specificity). To examine the substrate specificity of the SMVT, [^{3}H]biotin (10 nM) uptake was carried out in the presence of 1 mM biotin, its structural analogues, i.e., desthiolbiotin, biocytin, and biotin methyl ester, and some other carboxylic acids such as valeric acid, acetic acid, benzoic acid, and related vitamins pantothenic acid and lipoic acid. Biotin-saquinavir (biotin-conjugated saquinavir prodrug) was also applied for competitive inhibition of biotin uptake.

Unlabeled biotin or biotin-saquinavir was dissolved in DMSO to generate 10 mM stock solution. Then 25 μM solutions were made by diluting the stock solution with DPBS, and [^{3}H]biotin was added right before an experiment. The inhibition study was conducted according to the procedure described previously.

Transport Experiments. Permeability of [^{3}H]biotin (1.0 μCi/mL) across MDCK-MDR1 cells in the absence and presence of 50 μM biotin or biotin-saquinavir was determined with 12-well Transwell plates. Before each experiment, cells were grown on Transwell inserts. Cell monolayers were rinsed 3 times with DPBS pH 7.4 at 37 °C. Volumes of apical and basal chambers were 0.5 and 1.5 mL, respectively. Transport experiments were conducted for a period of 3 h. Samples (100 μL) were withdrawn from the receiver chamber at predetermined time points, i.e., 15, 30, 45, 60, 90, 120, 150, and 180 min, and replaced with equal volumes of fresh DPBS buffer solution to maintain sink conditions. Dilutions were taken into account for calculations of permeant concentrations. Each sample along with 5 mL of scintillation cocktail was then analyzed by a Beckman scintillation counter. All experiments were performed at 37 °C.

Transepithelial permeability of biotin-saquinavir in MDCK-MDR1, MDCK wild type (MDCK-WT), and Caco-2 cells was also determined. Samples (200 μL) in DPBS were withdrawn from the receiver chamber at predetermined time points i.e., 30, 60, 90, 120, 150, and 180 min. Samples were stored at − 80 °C until further analysis. Samples were then extracted with *tert*-butyl methyl ether. The ether layer was separated and then evaporated under vacuum. Then the dried samples were redissolved in water and analyzed by LC/MS/MS (Applied Biosystem/MDS SCIEX QTRAP).

Data Analysis. The kinetic parameters of biotin uptake were calculated by a modified Michaelis−Menten equation (eq 1), which takes into account the carrier-mediated process

(as described by the classical Michaelis−Menten equation) and the nonsaturable passive diffusion process.

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

v is the total rate of uptake, V_{max} is the maximum uptake for the carrier-mediated process, K_m is the Michaelis−Menten constant, and K_d is the rate for nonsaturable diffusion component. Data was fitted to eq 1 using the nonlinear least-squares regression analysis program (KaleidaGraph 3.5).

The cumulative amount transported (TR_{cum}) is calculated by eq 2:

$$TR_{cum} = A_n + \frac{V_n}{V_r}\sum_{i=0}^{n-1} A_i \quad (2)$$

A_n is the amount of drug measured in sample n, V_n is the volume of sample n, V_r is the volume of the receiver chamber, and A_i is the amount of drug at each predetermined time point.

Transepithelial permeability of [^{3}H]biotin or biotin-saquinavir was calculated by eq 3, respectively:

$$P_{A-B(orB-A)} = \frac{TR_{cum}}{dt}\frac{1}{A}\frac{1}{C_0} \quad (3)$$

TR_{cum}/dt is transport rate of [^{3}H]biotin or biotin-saquinavir, which is obtained from the slope of the transport profile. A is the surface area of the plate, and C_0 is the substrate concentration.

Statistical Analysis. All experiments were conducted at least in triplicate, and results were expressed as mean ± SD. Student's t test is used to detect statistical significance, and $P < 0.05$ is considered to be significant. Statistical comparisons were performed using the analysis of variance (GraphPad INSTAT, version 3.1).

RT-PCR Analysis. Total RNA was extracted from MDCK-MDR1 cells with Trizol reagent (Invitrogen) following manufacturer's instructions. Reverse transcription (RT) was performed according to standard protocol with 1 μg total RNA to obtain first strand cDNA. One microliter of cDNA was then introduced into PCR, and the product was subjected to gel electrophoresis with 3% agarose gel. The primers used for the amplification of SMVT were forward 5′-CGATTCAATAAAACTGTGCGAGT-3′ and reverse 5′-GGACAGCCACAGATCAAAGC-3′. The primers used were adopted from a published human SMVT cDNA sequence. PCR conditions were as follows: denaturation (94 °C, 45 s), annealing (58 °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 resultant product was sequenced from both directions by SeqWright with an automated Perkin-Elmer Applied Biosystems 3730x1 Prism TM DNA sequencer to establish its molecular identity.

Computer Analysis. Nucleotide sequence homology matching was performed by applying Basic Local Alignment Search Tool (BLAST) via on-line connection to the National Center of Biotechnology Information (NCBI) database. Multiple nucleotide sequence comparisons were made utilizing CLUSTAL W (1.81) multiple sequence alignment tool from Swiss-Prot.

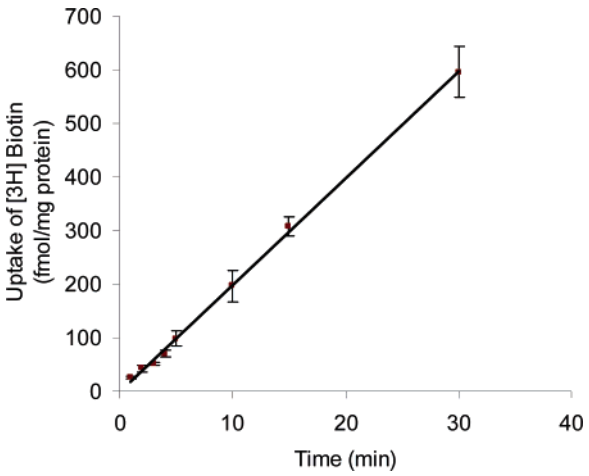


Figure 1. Time course of [^{3}H]biotin uptake in MDCK-MDR1 cells. Uptake of [^{3}H]biotin (10 nM) was measured in DPBS buffer (pH 7.4) at 37 °C. Data are shown as mean ± SD, $n = 3-6$. The linear equation is represented as $y = 20.0x - 1.57$ ($r^2 = 0.999$).

Results

Time Course. Time-dependent uptake of [^{3}H]biotin by MDCK-MDR1 cells was first determined. Figure 1 depicts the time course of intracellular biotin accumulation in MDCK-MDR1 cells at 37 °C. [^{3}H]Biotin (10 nM) uptake was linear for up to 30 min of incubation time ($r^2 = 0.999$) and occurred at a rate of 20.0 fmol min^{-1} (mg of protein)$^{-1}$. An incubation period of 5 min was selected for subsequent biotin uptake experiments unless otherwise mentioned.

Sodium Dependence. Na^+ dependence of biotin uptake was examined by replacing Na^+ in the incubation medium with an equimolar concentration of choline and K^+. Biotin uptake rates were 15.9 and 2.30 fmol min^{-1} (mg of protein)$^{-1}$ in the presence and absence of sodium, respectively (Figure 2). Replacing Na^+ in the incubation medium caused a 7-fold decrease in biotin uptake. It appears that the process of biotin uptake by MDCK-MDR1 cells is highly sodium dependent.

Temperature Dependence. Effect of temperature on the uptake of biotin by MDCK-MDR1 cells was studied. Initial uptake rates of biotin were 20.0 ± 1.53, 12.8 ± 0.49, and 7.29 ± 0.48 fmol min^{-1} (mg of protein)$^{-1}$ at 37 °C, 25 °C, and 4 °C, respectively. Uptake significantly diminished as incubation temperature was lowered, suggesting that the process is very sensitive to temperature. Uptake rate ($\ln(v)$) vs $1/T$ was plotted (Figure 3), and activation energy (E_a) was obtained as 5.10 kcal/mol.

Effect of Buffer pH and Metabolic Inhibitors. Figure 4 clearly indicates that the uptake of biotin was highest at pH 5.5 and decreased as the buffer pH was raised from 5.5 to 7.4. Then the rate was maintained the same as the buffer pH changed from 7.4 to 8.0.

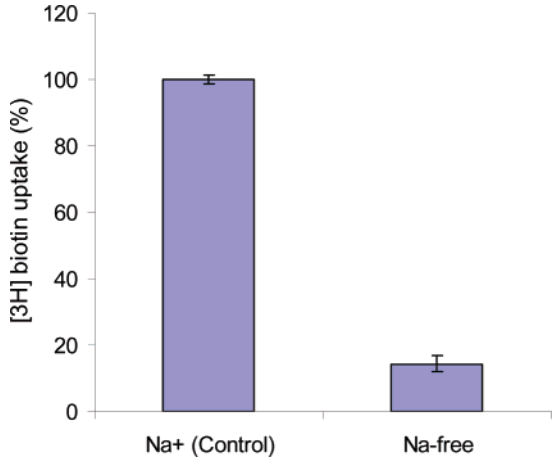


Figure 2. Effect of Na^+ on [^{3}H]biotin uptake in MDCK-MDR1 cells. Uptake of [^{3}H]biotin (10 nM) was measured in DPBS buffer (pH 7.4) with or without sodium for 5 min. Results are shown as mean ± SD, $n = 4-8$.

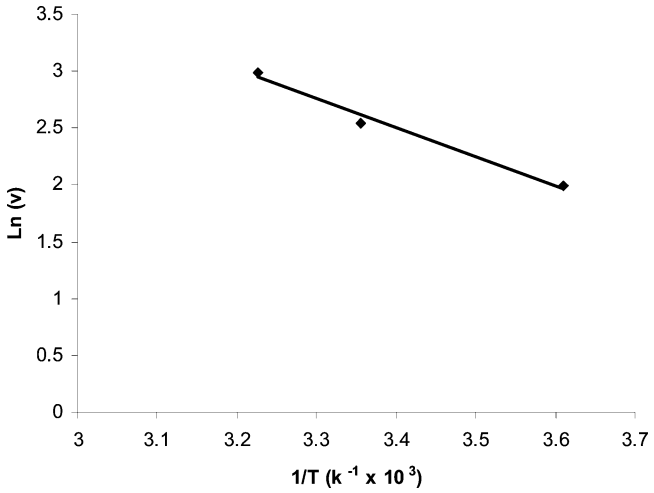


Figure 3. Arrhenius plot of the effect of temperature on [^{3}H]-biotin uptake by MDCK-MDR1 cells. Uptake of [^{3}H]biotin (10 nM) was measured in DPBS buffer (pH 7.4) at 37 °C. Data are shown as mean ± SD, $n = 3-6$.

To determine whether biotin uptake is energy dependent, the effect of metabolic inhibitors on the uptake process was examined by preincubating cells for 30 min with the metabolic inhibitors, i.e., 2,4-dinitrophenol (DNP), sodium azide, and ouabain (all at 1 mM). All three inhibitors did not show any significant inhibition to biotin uptake in MDCK-MDR1 cells, which suggests that the uptake of biotin is energy independent (data not shown).

Saturation Kinetics of Carrier-Mediated Mechanism for Biotin Uptake. [^{3}H]Biotin uptake in MDCK-MDR1 cells was determined as a function of substrate concentration in the range of 1−100 μM. The experiment was performed in the presence of Na^+ at 37 °C for 5 min. Uptake of biotin consisted of a major (saturable) carrier-mediated process and a minor nonsaturable component evident at higher concentrations (Figure 5). Uptake data was fitted to eq 1, and kinetic parameters K_m and V_{max} were determined to be 13.0 μM and 21.5 pmol min^{-1} (mg of protein)$^{-1}$. K_d had a value of 0.12 μL min^{-1} (mg of protein)$^{-1}$. Lineweaver−Burk transforma-

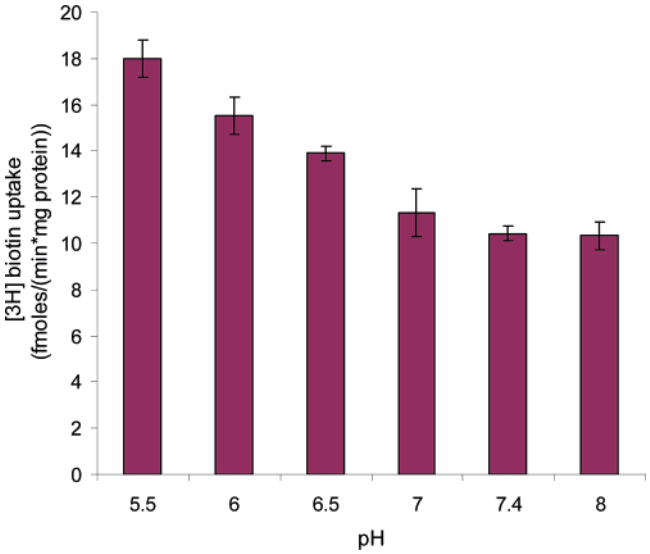


Figure 4. Effect of pH on [^{3}H]biotin uptake in MDCK-MDR1 cells. Uptake of [^{3}H]biotin (10 nM) was measured in DPBS buffer at different pH values (pH 5.5, 6.0, 6.5, 7.0, 7.4, 8.0) at 37 °C for 5 min. Results are expressed as mean ± SD, $n = 4-8$.

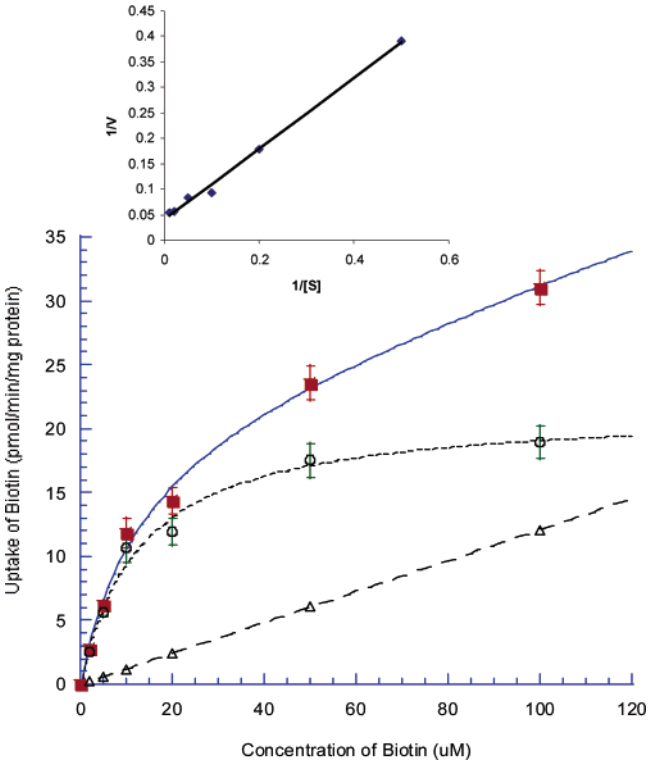


Figure 5. Saturation kinetics of [^{3}H]biotin uptake by MDCK-MDR1 cells. Cells were incubated at 37 °C in DPBS buffer (pH 7.4) for 5 min in the presence of different concentrations of biotin (1−100 μM). Results are expressed as mean ± SD, $n = 4-8$. (■) Total uptake. (○) Michaelis−Menten component. (△) Linear, nonsaturable component. Inset shows Lineweaver−Burk transformation of the data.

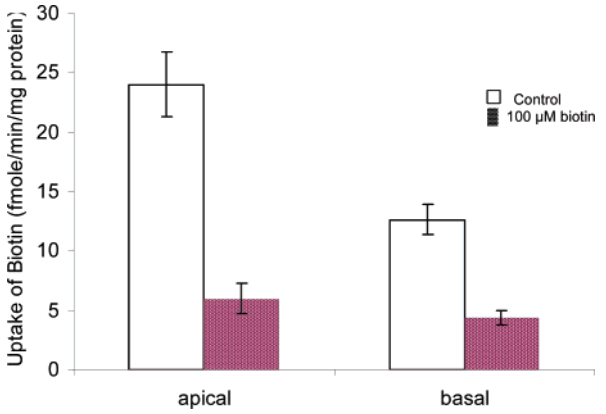


Figure 6. Apical and basal uptake of [^{3}H]biotin by MDCK-MDR1 cells. Uptake of [^{3}H]biotin (10 nM) was measured in DPBS buffer (pH 7.4) at 37 °C; 0.5 and 1.5 mL of [^{3}H]biotin solution was added to the apical and basal chambers of a 12-well Transwell plate for apical and basal uptake, respectively. The unfilled bars show the apical and basal uptake of [^{3}H]biotin in the absence of unlabeled biotin. The filled bars describe the apical and basal uptake of [^{3}H]biotin in the presence of 100 μM biotin. Data are shown as mean ± SD, n =3−6.

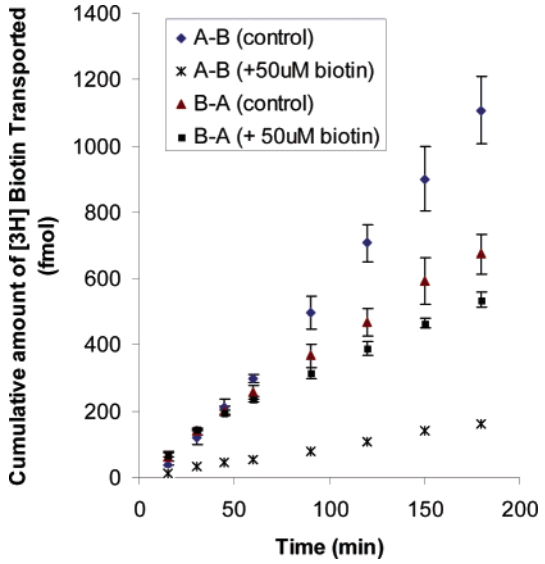


Figure 7. Transepithelial transport of [^{3}H]biotin in MDCK-MDR1 cells. Cumulative amount of transported [^{3}H]biotin (20 nM) was measured in DPBS buffer (pH 7.4) at 37 °C for 180 min in the absence and presence of 50 μM unlabeled biotin. Data are expressed as mean ± SD, n = 3−6. When the error bar is smaller than the symbol, it is not shown.

tion of the data pointed to the involvement of a single carrier in the uptake process (inset, Figure 5).

Polarized Distribution of SMVT in MDCK-MDR1 Cells. To investigate the polarized distribution of SMVT, uptake of [^{3}H]biotin on both apical and basal membrane in the absence and presence unlabeled biotin was determined (Figure 6). Transepithelial transport of [^{3}H]biotin (both A−B and B−A) in the absence and presence of unlabeled biotin was also examined (Figure 7). Amount of biotin uptake from basal membrane is half of that from the apical side. A 100 μM concentration of unlabeled biotin produced more inhibition on the apical uptake than that on the basal uptake. A−B permeability ((5.44 ± 0.55) × 10^{-6} cm/s) of [^{3}H]biotin was higher than B−A permeability ((3.07 ± 0.34) × 10^{-6} cm/s). In the presence of 50 μM biotin, A−B permeability diminished to a larger extent than B−A permeability, (5.92 ± 0.027) × 10^{-7} versus (2.27 ± 0.092) × 10^{-6} cm/s (Figure 7).

Substrate Specificity. To confirm the involvement of SMVT, we examined the substrate specificity of the transporter by inhibiting the uptake of [^{3}H]biotin (10 nM) with 1 mM biotin, its structural analogues, and related vitamins (Table 1). Uptake was significantly inhibited by unlabeled biotin, pantothenic acid, lipoic acid, desthiolbiotin, and valeric acid, but unaltered by other compounds, i.e., biocytin, biotin methyl ester, acetic acid, and benzoic acid. Lipoic acid caused maximum inhibition (93%) of biotin uptake.

We also examined the inhibition of different concentrations of biotin, pantothenic acid, and desthiolbiotin to the uptake of biotin (Table 2). It shows that the percentage of inhibition increased at higher incubator concentrations.

Inhibitory Effect of Biotin-Saquinavir. In the presence of 25 μM biotin and biotin-saquinavir, uptake of [^{3}H]biotin reduced to 36% and 72% of control, respectively (Figure 8). We also examined the effects of unlabeled biotin and biotin-saquinavir to the transport of [^{3}H]biotin across the MDCK-MDR1 cells. Both biotin and biotin-saquinavir (50 μM) exhibited significant inhibition to the transport of [^{3}H]-biotin from the apical to the basal side (A−B) (Figure 9). Apparent permeability (P_{A-B}) values were (5.44 ± 0.55) × 10^{-6}, (5.92 ± 0.027) × 10^{-7}, and (2.42 ± 0.13) × 10^{-6} cm/s for control and in the presence of biotin and biotin-saquinavir, respectively. P_{A-B} descended by 7- and 2-fold in the presence of biotin and biotin-saquinavir, respectively, confirming the presence of biotin carrier in MDCK-MDR1 cells.

Table 1. Uptake of [^{3}H]Biotin in MDCK-MDR1 Cells in the Presence of Various Inhibitors (1 mM)[a]

inhibitor (1 mM)	uptake as of control	P
control	100 ± 2.56	
biotin	9.03 ± 1.51	<0.01
pantothenic acid	9.12 ± 1.39	<0.01
lipoic acid	6.97 ± 0.82	<0.01
desthiolbiotin	15.61 ± 2.41	<0.01
valeric acid	60.39 ± 4.02	<0.01
biocytin	113.22 ± 3.43	ns[b]
biotin methyl ester	102.25 ± 4.82	ns
acetic acid	96.18 ± 6.76	ns
benzoic acid	112.65 ± 0.45	ns

[a] Uptake of [^{3}H]biotin (10 nM) was measured in DPBS buffer (pH 7.4) at 37 °C for 5 min in the presence of its structural analogues (1 mM). Data are shown as means ± SD, n = 4−8. P values were calculated with Student's t test and compared to the controls. [b] Not statistically significant at P < 0.05.

Transepithelial Permeability of Biotin-Saquinavir. Transepithelial permeability of biotin-saquinavir in MDCK-MDR1, MDCK-WT, and Caco-2 were determined (Table 3). A−B

Table 2. Concentration-Dependent Inhibition of [^{3}H]Biotin Uptake in MDCK-MDR1 Cells by Unlabeled Biotin, Pantothenic Acid, and Desthiolbiotin[a]

inhibitor	concn (μM)	uptake as of control	*P*
biotin	0	100 ± 4.85	
	10	51.50 ± 7.46	<0.01
	100	16.27 ± 0.47	<0.01
pantothenic acid	0	100 ± 5.02	
	10	27.22 ± 1.06	<0.01
	100	25.62 ± 2.06	<0.01
desthiolbiotin	0	100 ± 3.27	
	10	40.97 ± 4.29	<0.01
	100	16.67 ± 1.69	<0.01

[a] Uptake of [^{3}H]biotin (10 nM) was measured in DPBS buffer (pH 7.4) at 37 °C for 5 min in the presence of various concentrations of unlabeled biotin, pantothenic acid, and desthiolbiotin. Data are shown as mean ± SD, $n = 4-6$.

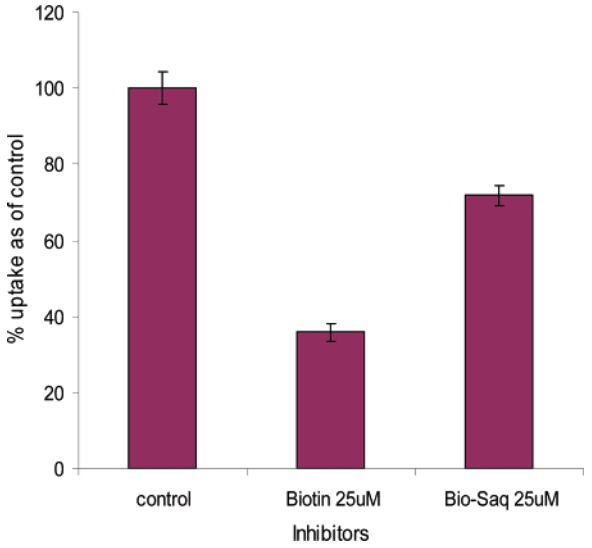


Figure 8. Inhibition of [^{3}H]biotin uptake in MDCK-MDR1 cells by unlabeled biotin or biotin-saquinavir. Cells were incubated at 37 °C in DPBS buffer (pH 7.4) for 5 min in the presence of 25 μM unlabeled biotin or biotin-saquinavir. Data are expressed as mean ± SD, $n = 4-8$.

permeability values of biotin-saquinavir are very similar: $(4.61 \pm 0.50) \times 10^{-7}$ cm/s in MDCK-MDR1, $(5.23 \pm 0.61) \times 10^{-7}$ cm/s in MDCK-WT, and $(3.90 \pm 0.36) \times 10^{-7}$ cm/s in Caco-2. A−B permeability of biotin-saquinavir in MDCK-WT is slightly higher than in MDCK-MDR1, which in turn is slightly higher than in Caco-2. However, B−A permeability of biotin-saquinavir in MDCK-MDR1 $((8.38 \pm 0.31) \times 10^{-7}$ cm/s) is slightly lower than that in Caco-2 $((10.68 \pm 0.59) \times 10^{-7}$ cm/s). The efflux ratio of biotin-saquinavir in Caco-2 is slightly higher than that in MDCK-MDR1. A−B permeability of biotin-saquinavir in both MDCK-MDR1 and Caco-2 cells is comparable to that of saquinavir $((4.63 \pm 0.235) \times 10^{-7}$ cm/s) in MDCK-MDR1 cells.[29] However, the efflux ratios of biotin-saquinavir in both Caco-2 and MDCK-MDR1 are much lower than those of saquinavir.[29]

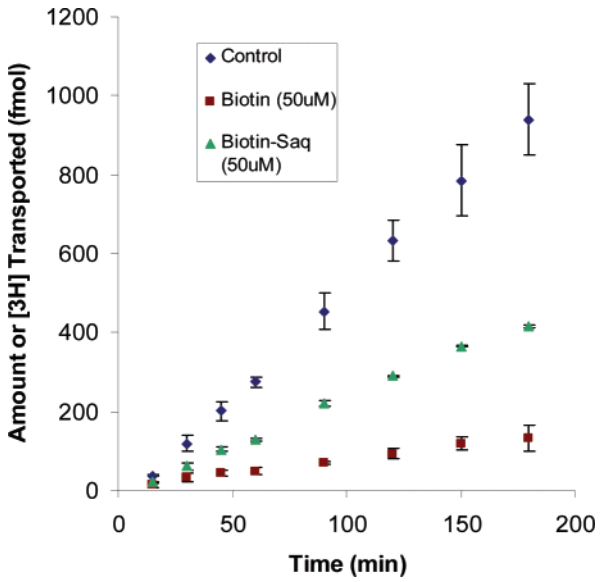


Figure 9. Inhibition of [^{3}H]biotin transport by unlabeled biotin or biotin-saquinavir across MDCK-MDR1 cells. Cumulative amount of transported [^{3}H]biotin (20 nM) was measured in DPBS buffer (pH 7.4) at 37 °C for 180 min in the presence of 50 μM unlabeled biotin and its prodrug biotin-saquinavir. Data are expressed as mean ± SD, $n = 3-6$. When the error bar is smaller than the symbol, it is not shown.

Table 3. Transepithelial Permeability of Biotin-Saquinavir in Different Cell Lines

	P_{appA-B} (cm/s, $\times 10^{-7}$)	P_{appB-A} (cm/s, $\times 10^{-7}$)	efflux index
MDCK-MDR1	4.61 (± 0.50)	8.38 (± 0.31)	1.82
MDCK−WT	5.23 (± 0.61)	*a*	
Caco-2	3.90 (± 0.36)	10.68 (± 0.59)	2.74

[a] Not determined.

Existence of the Second Biotin-Specific High Affinity Transporter. To examine the existence of a second biotin-specific high affinity transporter, we studied uptake at very low concentrations (0.1−10 nM) of biotin. Figure 10 clearly shows that biotin uptake is linear with concentration on the order of nanomoles per liter. We also examined inhibition of 50 nM unlabeled biotin, pantothenic acid, lipoic acid, and desthiolbiotin on the uptake of [^{3}H]biotin (2 nM). All four compounds in the nanomolar concentration range did not show any significant inhibition (Figure 11).

RT-PCR. The PCR product was analyzed by gel electrophoresis with 3% agarose gel. cDNA generated from total RNA isolated from MDCK-MDR1 cells was PCR amplified with the human SMVT primers. A product at 862 bp was obtained (Figure 12). The sequence obtained in both directions showed maximum homology with the human SMVT sequence at 862 bp (6001−6863) according to the BLAST search (NCBI) database, which confirmed the presence of

(29) Jain, R., Agarwal, S., Majumdar, S., Zhu, X., Pal, D., Mitra, A. K. Evasion of P-gp mediated cellular efflux and permeability enhancement of HIV-protease inhibitor saquinavir by prodrug modification. *Int. J. Pharm.* **2005**, *303*, 8−19.

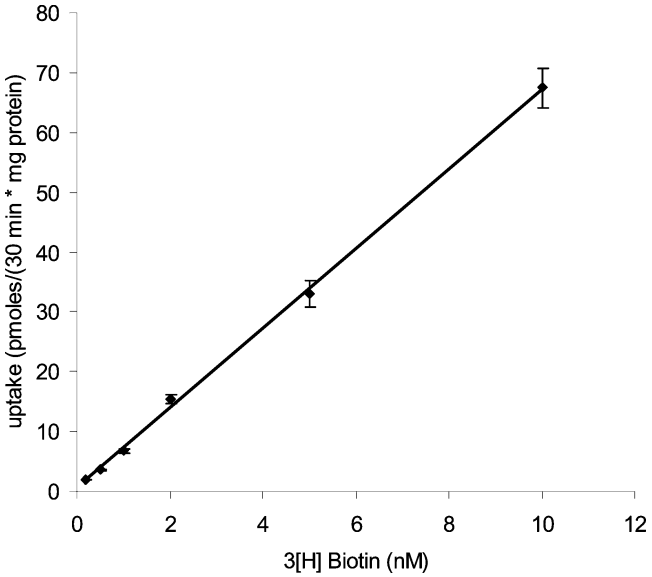


Figure 10. Uptake of [^{3}H]biotin in the nanomolar range by MDCK-MDR1 cells. Uptake of [^{3}H]biotin (0.2−10 nM) was measured in DPBS buffer (pH 7.4) at 37 °C for 30 min. No saturation was observed up to 10 nM concentration. Data are expressed as mean ± SD, $n = 4-6$.

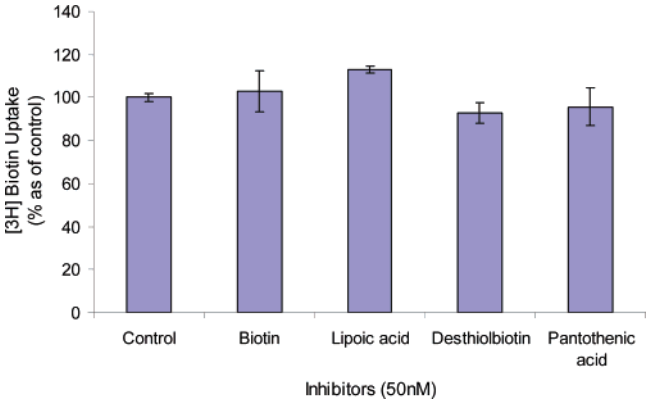


Figure 11. Inhibition of [^{3}H]biotin (2 nM) uptake in MDCK-MDR1 cells in the presence of 50 nM unlabeled biotin and its structural analogues. Results are expressed as mean ± SD, $n = 4-6$.

SMVT in MDCK-MDR1 cells. Thus a sodium-dependent multivitamin transporter (SMVT) is expressed in MDCK-MDR1 cells.

Disscusion

The primary objective of this study was to investigate the functional and molecular expression of the sodium-dependent multivitamin transporter (SMVT) in the MDCK-MDR1 cells. This cell line has been selected because it can provide an alternative to the Caco-2 model for high throughput screening in drug discovery.[26−28] This vitamin carrier system may be targeted to enhance active drug uptake and transport.[21−24] To establish the suitability of the MDCK-MDR1 cell line as an in vitro model for permeability studies of biotin-

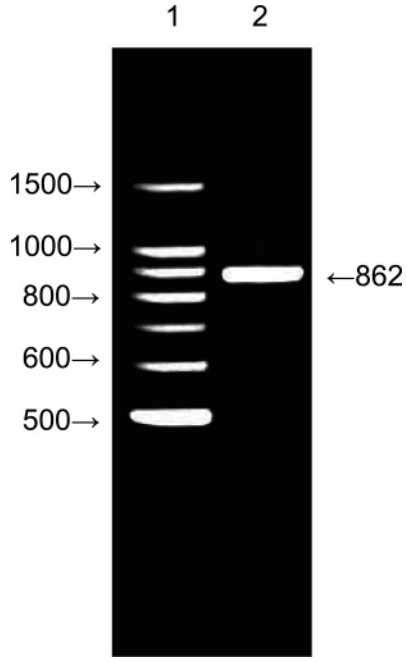


Figure 12. RT-PCR showing the expression of SMVT in MDCK-MDR1 cells. Lane 1: Standards. Lane 2: PCR product obtained from total RNA isolated from MDCK-MDR1 cells with hSMVT-specific primers. Samples were analyzed on a 3% agarose gel. An 862 base pair band is very prominent.

conjugated prodrugs, transepithelial transport of biotin-saquinavir in MDCK-MDR1 and related cell lines, i.e., MDCK-WT and Caco-2, were carried out and permeability values were compared.

The functional mechanism involved in the uptake and transport of biotin in the MDCK-MDR1 cells was first delineated. The uptake process was strongly Na^+ dependent (Figure 2). Variations in uptake rates at different temperatures suggest that the process is highly temperature dependent (Figure 3). However, no significant inhibition of biotin uptake by MDCK-MDR1 cells was observed in the presence of metabolic inhibitors, i.e., sodium azide, ouabain, and dinitrophenol, indicating energy independence. Our result was different from the findings of Balamurugan et al.[12] on human proximal tubular epithelial cells (HK-2 cells), which showed that the biotin uptake was energy dependent. A difference in the energy-dependence parameter may suggest a rather different energetics in this cell line. Biotin uptake was modestly lower when pH varied from 5.5 to 7.4, and was almost same within a pH range of 7.4−8.0 (Figure 4). Such a pH effect may be caused by the ionic nature of biotin not from the H^+ gradient.[13] A larger fraction of neutral biotin component at lower pH may cause higher biotin uptake since neutral species usually generate higher permeability across the lipid bilayer of the cell membrane.

Uptake of biotin by MDCK-MDR1 cells was found to be concentration dependent and saturable at the micromolar range with a K_m of 13.0 μM (Figure 5), which is almost the same as the value (12.16 μM) reported for the HK-2 cells,[12] suggesting a carrier-mediated uptake over the micromolar

range. The process was strongly inhibited by unlabeled biotin and other two vitamins, pantothenic acid and lipoic acid, known SMVT substrates (Table 1). One millimolar biotin, pantothenic acid, and lipoic acid produced almost 90% inhibition, suggesting that the biotin uptake was mediated by the same carrier, i.e., sodium-dependent multivitamin transporter (SMVT). Inhibition of [^{3}H]biotin transport across the apical to the basal side in the presence of excess unlabeled biotin further proves that SMVT transporter facilitates biotin transport across the cell membrane (Figure 7). The same concentration of biotin analogue, i.e., desthiolbiotin, produced a similar extent of inhibition. However, the same concentration of valeric acid produced much less inhibition than biotin. Acetic acid and benzoic acid did not produce significant inhibition of biotin uptake (Table 1), which proves that biotin uptake is not mediated by the monocarboxyl acid transporter (MCT). No significant inhibition on biotin uptake was produced by biocytin and biotin methyl ester (Table 1). These results suggest that the long side chain with a free carboxyl moiety of the biotin molecule may be required for recognition and interaction with SMVT. Similar structural requirements have been reported for SMVT.[13,19−20]

Apical and basal uptake and bidirectional transepithelial transport of [^{3}H]biotin in MDCK-MDR1 cells suggest that biotin uptake is higher from the apical membrane than from the basal membrane. A−B permeability of biotin is greater than B−A permeability. The same amount of unlabeled biotin produced more inhibition on the apical uptake and A−B transport than basal uptake and B−A transport, respectively. These results confirm polarized distribution of SMVT at the apical side relative to the basal membrane.

Our biotin-saquinavir prodrug produced significant inhibition of the uptake and transport of [^{3}H]biotin (Figures 8 and 9), which indicates that this prodrug can be recognized by SMVT transporter. The inhibition of biotin-saquinavir was less than that of biotin itself since biotin-saquinavir may not have similar affinity for SMVT relative to biotin.

A−B permeability of biotin-saquinavir in MDCK-WT is slightly higher than in MDCK-MDR1 and Caco-2 cells, which in turn is slightly higher than in Caco-2. The efflux ratio of biotin-saquinavir in MDCK-MDR1 is less than that in Caco-2. The efflux ratios of biotin-saquinavir in both MDCK-MDR1 and Caco-2 are significantly lower than those of saquinavir,[29] which suggests that biotin-saquinavir is a significantly poorer P-gp substrate than saquinavir itself.

A second biotin-specific high affinity transport system active within the nanomolar concentration range has been reported in skin and PBMC cells.[14−15] From uptake results shown in Figures 10 and 11, the cumulative amount of biotin uptake by MDCK-MDR1 cells was linear as a function of biotin concentration over the nanomolar range. Fifty nanomolar biotin, lipoic acid, pantothenic acid, and desthiolbiotin did not produce significant inhibition of biotin uptake, indicating that the second biotin-specific high affinity system active in the nanomolar range is not functional in the MDCK-MDR1 cells.

Finally, the RT-PCR result further confirms the presence of SMVT in the MDCK-MDR1 cells. The sequence obtained in both directions at a band of 862 bp exhibited maximum homology with the human SMVT sequence at 862 bp (6001−6863) and also partially matched with that of canis familiaris (dog) when the BLAST of NCBI database was searched. Balamurugan et al. reported a band of 1636 bp for the HK-2 cells.[12] Such a difference may be explained by variants in SMVT genes and/or transcript variants of SMVT genes.

Conclusion

In conclusion, this study demonstrates functional evidence of a sodium-dependent multivitamin carrier system, SMVT, in the MDCK-MDR1 cell line. RT-PCR further provides molecular evidence for the presence of SMVT in the MDCK-MDR1 cells. Biotin-saquinavir produced significant inhibition in the uptake and transport of [^{3}H]biotin, and the conjugate was recognized by SMVT transporter. The permeability of biotin-saquinavir in MDCK-MDR1, MDCK-WT, and Caco-2 was very similar. The MDCK-MDR1 cell line may thus be employed as a valuable in vitro tool for screening the permeability of biotin-conjugated protease inhibitors such as saquinavir. The efflux ratio of biotin-saquinavir was much less than that of saquinavir, which suggests that the prodrug is a much poorer P-gp substrate than saquinavir.

Acknowledgment. This study was supported by NIH Grant R01 GM 64320-03. The donation of saquinavir mesylate by Hoffmann-La Roche is highly appreciated. The authors would like to thank Balasubrahmanyam Budda for his help with RT-PCR and Zhiying Wang for her help with growing MDCK-MDR1, MDCK-WT, and Caco-2 cells.

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