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Targeted lipid based drug conjugates: A novel strategy for drug delivery

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ABSTRACT

A majority of studies involving prodrugs are directed to overcome low bioavailability of the parent drug. The aim of this study is to increase the bioavailability of acyclovir (ACV) by designing a novel prodrug delivery system which is more lipophilic, and at the same time site specific. In this study, a lipid raft has been conjugated to the parent drug molecule to impart lipophilicity. Simultaneously a targeting moiety that can be recognized by a specific transporter/receptor in the cell membrane has also been tethered to the other terminal of lipid raft. Targeted lipid prodrugs i.e., biotin-ricinoleicacid-acyclovir (B-R-ACV) and biotin-12hydroxystearicacid-acyclovir (B-12HS-ACV) were synthesized with ricinoleicacid and 12hydroxystearicacid as the lipophilic rafts and biotin as the targeting moiety. Biotin-ACV (B-ACV), ricinoleicacid-ACV (R-ACV) and 12hydroxystearicacid-ACV (12HS-ACV) were also synthesized to delineate the individual effects of the targeting and the lipid moieties. Cellular accumulation studies were performed in confluent MDCK-MDR1 and Caco-2 cells. The targeted lipid prodrugs B-R-ACV and B-12HS-ACV exhibited much higher cellular accumulation than B-ACV, R-ACV and 12HS-ACV in both cell lines. This result indicates that both the targeting and the lipid moiety act synergistically toward cellular uptake. The biotin conjugated prodrugs caused a decrease in the uptake of [^{3}H] biotin suggesting the role of sodium dependent multivitamin transporter (SMVT) in uptake. The affinity of these targeted lipid prodrugs toward SMVT was studied in MDCK-MDR1 cells. Both the targeted lipid prodrugs B-R-ACV (20.25 ± 1.74 μM) and B-12HS-ACV (23.99 ± 3.20 μM) demonstrated higher affinity towards SMVT than B-ACV (30.90 ± 4.19 μM). Further, dose dependent studies revealed a concentration dependent inhibitory effect on [^{3}H] biotin uptake in the presence of biotinylated prodrugs. Transepithelial transport studies showed lowering of [^{3}H] biotin permeability in the presence of biotin and biotinylated prodrugs, further indicating a carrier mediated translocation by SMVT. Overall, results from these studies clearly suggest that these biotinylated lipid prodrugs of ACV possess enhanced affinity towards SMVT. These prodrugs appear to be potential candidates for the treatment of oral and ocular herpes virus infections, because of higher expression of SMVT on intestinal and corneal epithelial cells. In conclusion we hypothesize that our novel prodrug design strategy may help in higher absorption of hydrophilic parent drug. Moreover, this novel prodrug design can result in higher cell permeability of hydrophilic therapeutics such as genes, siRNA, antisense RNA, DNA, oligonucleotides, peptides and proteins.

1. Introduction

Acyclovir [ACV, 2-amino-9-((2-hydroxyethoxy) methyl)-1H-purin-6(9H)-one] is a guanosine analogue with excellent antiviral activity. Since its discovery, ACV has been indicated for the treatment of herpes simplex virus (HSV) and herpes zoster (shingles) infections. The compound is phosphorylated by viral thymidine kinases to ACV monophosphate, which is subsequently converted by cellular kinases to active ACV triphosphate. ACV triphosphate is then incorporated into viral DNA, inhibiting the activity of DNA polymerase and leading to chain termination (Piret and Boivin, 2011; Reardon and Spector, 1989; Wilson et al., 2009). ACV suffers from limited aqueous solubility and low oral bioavailability (15–30%), resulting in poor accumulation at the target site. A wide variety of strategies have been investigated to enhance cellular absorption among which transporter targeted prodrug strategy has been a promising approach. In our laboratory, the functional characteristics of various transporters have been exploited for targeted drug delivery. These include amino acid transporters (Anand et al., 2004; Jain-Vakkalagadda et al., 2003), peptide transporter (PEPT1 and PEPT2) (Anand et al., 2003a; Anand and Mitra, 2002), sodium dependent multivitamin transporter (SMVT) (Janoria et al., 2006,

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2009; Luo et al., 2006), sodium dependent vitamin C transporter (SVCT1 and SVCT2) (Luo et al., 2008; Talluri et al., 2006), riboflavin transporter (Hariharan et al., 2006), nucleoside and nucleobase transporters (Majumdar et al., 2003a,b). Valacyclovir (VACV), an amino acid ester prodrug of ACV has been shown to increase the oral bioavailability of ACV by 3- to 5-fold (Balimane et al., 1998; de Vrueh et al., 1998; Guo et al., 1999; Jacobson, 1993; Perry and Faulds, 1996; Soul-Lawton et al., 1995; Talluri et al., 2008).

SMVT is primarily responsible for the uptake of vitamins such as biotin, pantothenic acid and lipoate in epithelial cells (Chatterjee et al., 1999; Prasad et al., 1999; Vadlapudi et al., 2012). Biotin, a water soluble vitamin is essential for normal cellular growth. Absorption of biotin by SMVT is a pH dependent process. Significant amount of biotin absorption through SMVT has been reported in major tissues such as cornea, retina, kidney, intestine, liver and placenta (Balamurugan et al., 2003; Janoria et al., 2006, 2009; Luo et al., 2006; Prasad and Ganapathy, 2000; Prasad et al., 1998; Said, 1999; Said et al., 1987). SMVT has been utilized for delivery of poorly permeable drugs by conjugating biotin as the targeting moiety (Gunaseelan et al., 2004; Minko et al., 2002; Ramanathan et al., 2001b). Ramanathan et al. utilized this transporter to improve the intestinal absorption of peptides. Interaction of the PEG–biotin conjugates with SMVT has also been reported (Ramanathan et al., 2001b). Other conjugates like CPT–PEG–biotin showed enhanced anticancer activity relative to the parent drug camptothecin (CPT) in multidrug-resistant human ovarian carcinoma cells (Minko et al., 2002). Biotin conjugated nonapeptide R.I.-K (biotin)-Tat9 exhibited three times higher permeability in comparison to non-biotinylated R.I.-K-Tat9 across Caco-2 cell monolayers (Ramanathan et al., 2001a).

The apparent affinity constant (K_m) values of SMVT substrates are usually in the low micro molar range (Ma et al., 1994; Said, 1999; Said et al., 1987, 1992, 1994, 1998). Such low K_m values result in the saturation of the transporter which limits the dose of drug molecule that can be delivered through this carrier. To overcome this constraint, we have investigated the possibility of utilizing lipid-raft based drug conjugates to maximize the amount of drug transport. Biological membranes are lipophilic and hence only relatively lipophilic molecules can penetrate such membranes. It has been shown that the lipid moieties conjugated to drugs enhanced the absorption of drug resulting in higher oral bioavailability (Trevaskis et al., 2008). Acyclovir diphosphate dimyristoylglycerol (ACVDP-DG), a lipid prodrug of ACV was shown to be very active against HSV-1 and HSV-2, ACV-resistant strains of HSV and human cytomegalovirus (CMV) (Hostetler et al., 1993). Such lipid prodrugs had shown prolonged antiviral activity against HSV-1 retinitis in a rabbit model (Taskintuna et al., 1997). 1-O-hexadecylpropanediol-3-P-ACV, an orally bioavailable lipid prodrug of ACV is highly effective against acute HSV-1 infection in mice. It was also found to be active against CMV infections in vitro due to its ability to bypass thymidine kinases (Beadle et al., 2000). Recently, hexadecyloxypropyl esters of cidofovir and (S)-HPMPA have been synthesized. These lipid prodrugs were readily absorbed and converted intracellularly to their respective diphosphates following oral administration. These prodrugs are also orally active in animal models of viral infection (Beadle, 2007).

Previous work from our laboratory suggested that lipid prodrug diffuses readily across the cell membrane by facilitated diffusion whereas transporter/receptor targeted prodrug translocates compounds across the cell membrane via active transport. Both approaches have individually shown marginal improvement in cellular uptake. However, our current approach combines both lipid and transporter/receptor targeted delivery to generate synergistic effect. The lipid raft facilitates enhanced interaction of prodrug with membrane transporters/receptors probably assisting docking of the targeted ligand into the binding domain of transporter/receptor protein. The net effect is rapid translocation of the cargo across cell membrane. Biotinylated lipid prodrugs with different lipid rafts conjugated to ACV have been synthesized. These prodrugs include biotin-ricinoleicacid-ACV (B-R-ACV) and biotin-12hydroxystearicacid-ACV (B-12HS-ACV). To delineate the individual effects of the targeting and the lipid moieties, biotin-ACV (B-ACV), ricinoleicacid-ACV(R-ACV) and 12hydroxystearicacid-ACV (12HS-ACV) have also been synthesized. Emphasis was placed on the rate of cellular accumulation of these prodrugs for enhanced absorption of the parent drug, ACV. These compounds were evaluated for their ability to be translocated by SMVT across Caco-2 and MDCK-MDR1 cell lines. These model *in vitro* cell lines have been extensively employed to delineate compounds that may be recognized by SMVT (Balamurugan et al., 2003; Luo et al., 2006).

2. Materials and methods

2.1. Materials

ACV is a gift from GlaxoSmithKline (Research Triangle Park, NC). Biotin, ricinoleicacid and 12hydroxystearic acid were purchased from Sigma–Aldrich (St. Louis, MO). [^{3}H] biotin (60 Ci/mmol) was procured from Perkin-Elmer Life Science, Inc. (Boston, MA). Madin–Darby canine kidney cells transfected with human *mdr1* gene (MDCK-MDR1) were a generous gift from Drs. Schinkel and P. Borst (The Netherlands Cancer Institute, Amsterdam). Human colon carcinoma derived cells (Caco-2) were obtained from American Type Culture Collection (Manassas, VA). The growth medium, Dulbecco's modified Eagle's medium (DMEM) and TrypLE™ Express were obtained from Invitrogen (Carlsbad, CA). Nonessential amino acids, penicillin, streptomycin, sodium bicarbonate and HEPES were purchased from Sigma–Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). Culture flasks (75 cm^2 growth area), 12-well plates (3.8 cm^2 growth area per well) and 96-well plates (0.32 cm^2 growth area per well) were procured from Costar (Cambridge, MA). The buffer components and solvents were obtained from Fisher Scientific Co. (Fair Lawn, NJ). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO) and used without further purification.

2.2. Synthesis

Synthesis of ricinoleicacid-ACV (R-ACV). Ricinoleicacid (100 mg, 0.33 mmol) is dissolved in dimethyl formamide (DMF) (2 ml), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (126 mg, 0.66 mmol) is added and stirred for 1 h at room temperature to activate the carboxyl group of ricinoleicacid. In a separate reaction flask, ACV (110 mg, 0.49 mmol) is dissolved in DMF, 4-dimethylaminopyridine (DMAP) (60 mg, 0.49 mmol) is added and stirred for 10 min at room temperature to activate the hydroxyl group of ACV. This mixture is then added to the reaction vessel containing ricinoleicacid through a syringe and is stirred continuously for 72 h. Small portions of the reaction mixture are taken out and injected into LC/MS to ensure the complete conversion of the starting material to the product. The reaction mixture is then filtered and evaporated at room temperature under reduced pressure to generate crude product. The product R-ACV is then purified by silica gel column chromatography using 6% methanol/dichloromethane (MeOH/DCM) as eluent. The yield is approximately 47%. The synthetic scheme has been summarized in Scheme 1.

Synthesis of biotin-ricinoleicacid-ACV (B-R-ACV). R-ACV (30 mg, 0.05 mmol) is dissolved in DMF; DMAP (10 mg, 0.075 mmol) is added and stirred for 10 min at room temperature. In a separate reaction flask, biotin (29 mg, 0.11 mmol) is dissolved in DMF

(1 ml); EDC (23 mg, 0.11 mmol) is added and stirred for 1 h. This mixture is added into the reaction containing R-ACV through a syringe and is stirred continuously for 72 h. The complete conversion of the starting material to product is ensured by injecting small portion of the reaction mixture into LC/MS. The reaction mixture is filtered and evaporated under reduced pressure to generate crude product. The product B-R-ACV is purified by silica gel column chromatography using 20% MeOH/DCM as eluent. The yield is approximately 79%. The synthetic scheme has been summarized in Scheme 1.

Synthesis of 12hydroxystearicacid-ACV (12HS-ACV) and biotin-12hydroxystearicacid-ACV (B-12HS-ACV). 12HS-ACV and B-12HS-ACV are synthesized following the same procedure used for the synthesis of R-ACV and B-R-ACV respectively. The yield of B-12HS-ACV is approximately 78%. The synthetic scheme has been summarized in Scheme 2.

Synthesis of biotin-ACV (B-ACV). Biotin (100 mg, 0.40 mmol) is dissolved in DMF (2 ml), EDC (152 mg, 0.80 mmol) is added and stirred for 1 h. In a separate reaction flask ACV (184 mg, 0.80 mmol) is dissolved in DMF, DMAP (58 mg, 0.48 mmol) is added and stirred for 10 min at room temperature. This mixture is then added into the reaction containing biotin through a syringe and is stirred continuously for 72 h. The complete conversion of the starting material to product is ensured and the solvent is evaporated to yield the crude product. The product B-ACV is purified by silica gel column chromatography using 10% MeOH/DCM as eluent. The yield is approximately 78%. The synthetic scheme has been summarized in Scheme 3.

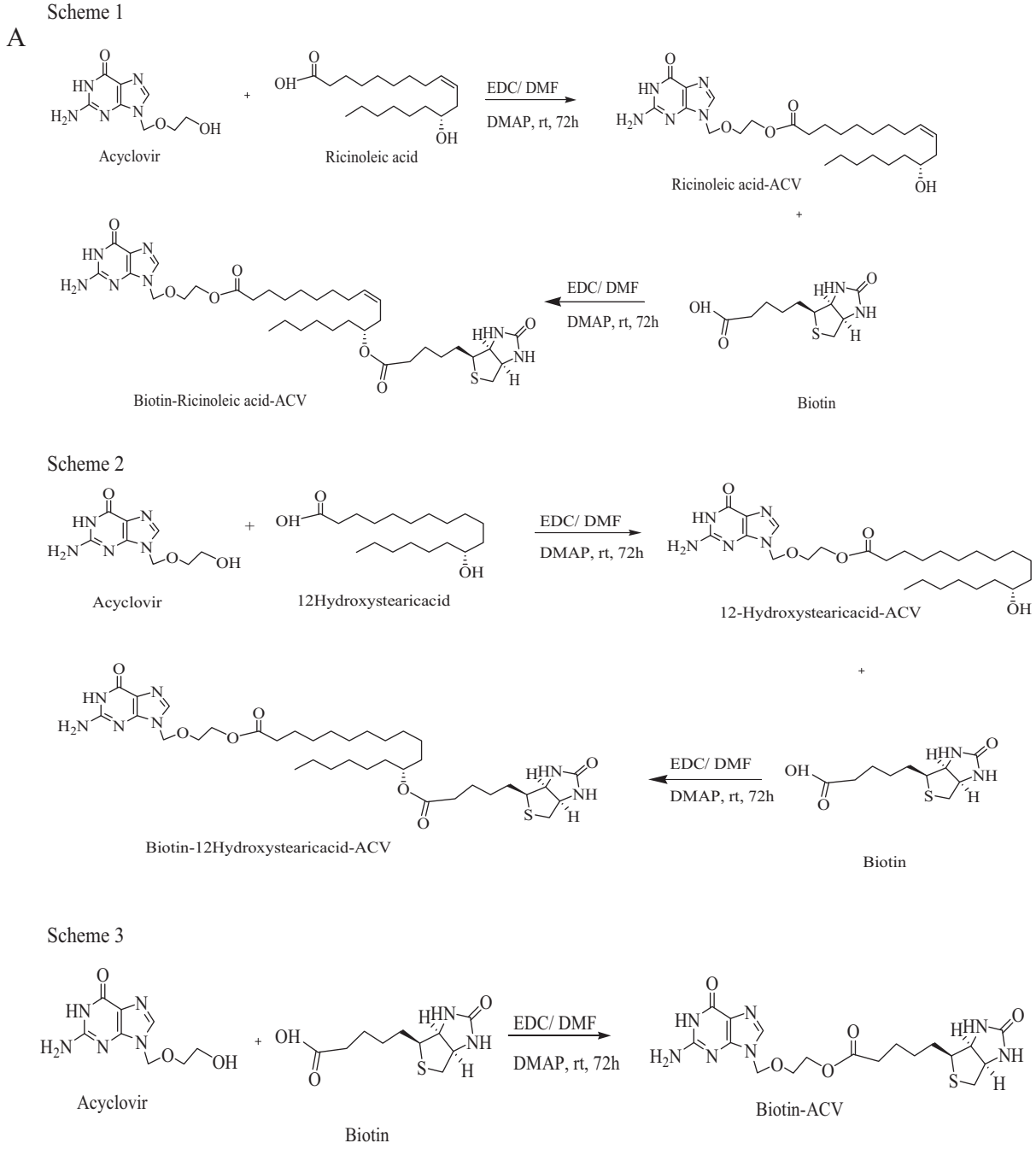

Fig. 1. (A) Scheme 1: Synthesis of ricinoleicacid-ACV and biotin-ricinoleicacid-ACV. Scheme 2: Synthesis of 12hydroxystearicacid-ACV and biotin-12hydroxystearicacid-ACV. Scheme 3: Synthesis of biotin-ACV. (B) The percent yield, mass and NMR (both ^{1}H NMR and ^{13}C NMR) spectra for all the synthesized prodrugs.

B

Ricinoleicacid-ACV(R-ACV): White solid, Yield 47%; LC/MS(M/z): 506.5; ^{1}HNMR(DMSO-d_6): δ 0.83 - 0.86 (t, J = 7 Hz, 3H), 1.23 - 1.47 (m, 20H), 1.97 - 2.10 (m, 4H), 3.36 (brs, 5H), 3.65 – 3.67 (m, 2H), 4.07 – 4.09 (m, 2H), 5.35 – 5.39 (m, 4H), 6.51 – 6.59 (m, 3H), 7.81 (s, 1H), 8.09 - 8.10(m, 1H); ^{13}CNMR(DMSO-d_6): 13.96, 22.09, 24.37, 25.19, 26.82, 28.40, 28.53, 28.57, 28.88, 29.04, 31.36, 33.29, 35.19, 36.47, 62.56, 66.55, 69.80, 71.80, 116.50, 126.63, 130.51, 137.66, 149.20, 151.42, 156.78, 172.80.

Biotin-Ricinoleicacid-ACV(B-R-ACV): White solid, Yield 79%; LC/MS(M/z): 732.3; ^{1}HNMR(DMSO-d_6): δ 0.82 - 0.85 (t, J = 7 Hz, 3H), 1.23 - 1.65 (m, 18H), 1.96 - 2.01 (m, 1H), 2.17 - 2.31 (m, 5H), 2.34 (brs, 1H), 2.55 - 2.60 (m, 2H), 2.79 - 2.83 (m, 2H), 2.92 - 3.02 (m, 4H), 3.05 - 3.23 (m, 3H), 3.65 - 3.67(m, 2H), 4.06 - 4.14 (m, 4H), 4.29 - 4.33 (m, 2H), 5.26 - 5.30 (m, 1H), 5.35 (s, 2H), 5.43 - 5.47(m, 1H), 6.41 - 6.45 (m, 3H), 6.64 (brs, 1H), 6.87 (brs, 1H), 8.11 (s, 1H); ^{13}CNMR(DMSO-d_6): 13.06, 22.03, 24.39, 24.70, 24.78, 26.75, 28.09, 28.21, 28.48, 28.53, 28.58, 28.98, 31.16, 31.53, 33.07, 33.31, 33.67, 33.91, 37.91, 55.46, 59.24, 61.10, 62.59, 66.56, 71.79, 72.95, 116.45, 124.47, 132.21, 137.55, 147.87, 154.27, 156.75, 162.79, 172.53, 172.85.

Biotin-12Hydroxystearicacid-ACV(B-12HS-ACV): White solid, Yield 78%; LC/MS(M/z): 734.5; ^{1}HNMR(CD_3OD): δ 0.88 - 0.91 (t, J = 7 Hz, 3H), 1.28 - 1.73 (m, 27H), 2.16 - 2.26 (m, 5H), 2.31 – 2.35 (m, 2H), 2.67 – 2.72 (m, 2H), 2.90 - 2.94 (m, 2H), 3.19 – 3.23 (m, 2H), 3.77 – 3.79 (m, 2H), 4.16 – 4.18 (m, 2H), 4.29 – 4.32 (m, 2H), 4.46 – 4.51 (m, 2H), 5.47 (s, 2H), 7.84 (s, 1H); ^{13}CNMR(CD_3OD): 13.93, 22.02, 24.38, 24.76, 26.03, 28.07, 28.11, 28.41, 28.49, 28.64, 28.69, 28.85, 28.90, 31.16, 31.30, 33.60, 33.66, 37.34, 55.42, 59.60, 59.20, 61.07, 62.58, 66.49, 71.68, 73.08, 116.36, 137.14, 151.55, 154.99, 162.71, 162.78, 172.62, 172.84.

Biotin-ACV (B-ACV): White solid, Yield 78%; LC/MS(M/z): 452.1; ^{1}HNMR(DMSO-d_6): δ 1.24 – 1.35 (m, 2H), 1.39 - 1.51 (m, 3H), 1.54 - 1.63 (m, 1H), 2.17 - 2.24 (m, 2H), 2.55 - 2.58 (m, 1H), 2.79 – 2.84 (m, 1H), 3.05 – 3.09 (m, 1H), 3.64 – 3.67 (m, 2H), 4.07 – 4.15 (m, 3H), 4.29 –4.32 (m, 1H), 5.34 (s, 2H), 6.37 (brs, 1H), 6.43 (brs, 1H), 6.55 (brs, 1H), 7.81 (s, 1H), 10.69 (brs, 1H); ^{13}C NMR(DMSO-d_6): 24.44, 27.96, 33.17, 55.38, 59.20, 61.06, 62.63, 66.59, 71.83, 116.48, 137.74, 151.45, 153.95, 156.83, 162.76, 172.79.

Fig. 1. (*Continued*).

All the reactions are run under inert atmosphere. The prodrugs are characterized by ^{1}H NMR, ^{13}C NMR and LC/MS. ^{1}H and ^{13}C NMR spectra are recorded using tetra methyl silane as an internal standard on Varian Mercury 400 Plus spectrometer. Chemical shifts (δ) are reported in parts per million relative to the NMR solvent signal (CD_3OD, 3.31 ppm for proton and 49.15 ppm for carbon NMR spectra; DMSO-d_6, 2.51 ppm for proton and 39.30 ppm for carbon NMR). A hybrid triple quadrupole linear ion trap mass spectrometer (QTRAP® LC/MS/MS mass spectrometer, API 3200, Applied Biosystems/MDS Sciex, Foster City, CA, USA) under enhanced mass (EMS) mode is used for carrying out the mass analysis. Electrospray ionization (ESI) is utilized as an ion source and operated in positive and negative ion mode.

2.3. Cell culture

Caco-2 (passage numbers 25–30) and MDCK-MDR1 cells (passage numbers 10–15) are utilized in the studies. Cells are grown at 37 °C in an incubator with 95% air and 5% CO_2. Cells are maintained in 75 cm^2 culture flasks using DMEM with 29 mM sodium bicarbonate, 20 mM HEPES, 100 μg/ml streptomycin, 100 μg/ml of penicillin, 10% FBS (heat inactivated) and 1% nonessential amino acids at pH 7.4. The medium is changed every alternate day. After reaching 80% confluency, cells are passaged using TrypLE™ Express (superior replacement for trypsin). Cells are subsequently plated in 12-well uptake plates at a density of 250,000 cells/well and in 96-well plates at a density of 10,000 cells/well. Cells are grown in a similar way and utilized for further studies.

2.4. Preparation of drug solutions

Concentrated stock solutions of all the drugs/prodrugs are prepared in dimethyl sulfoxide (DMSO). Test solutions are then prepared by adding aliquots from the respective stock solution and diluted with Dulbecco's Phosphate Buffered Saline (DPBS) (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 $MgSO_4$, 20 mM HEPES and 5 mM glucose) to achieve required concentration when needed. The final concentration of DMSO in all the drug solutions does not exceed 1% (v/v).

2.5. Cellular accumulation studies

Cellular accumulation studies are performed on MDCK-MDR1 and Caco-2 cell monolayers after growing them for 7 and 21 days, respectively. The medium is aspirated and cells are rinsed 3 times with DPBS. Uptake is initiated by adding 1 ml drug solution into each well and incubated for a period of 30 min. After incubation, drug solutions are removed and uptake is terminated with ice cold stop solution (200 mM KCl and 2 mM HEPES). Cells are lysed overnight at −80 °C in 500 μl cremophore water (2 drops of cremophore gel in 50 ml of deionized water) in each well. Samples are then analyzed with LC–MS/MS and the rate of uptake is normalized to the protein content of each well. The amount of protein in the cell lysate is estimated with BioRad protein estimation kit (BioRad, Hercules, CA) using bovine serum albumin as an internal standard. A similar procedure is adopted to determine cellular accumulation of the prodrugs in the presence of excess biotin to delineate the interaction of the prodrugs with SMVT (substrate specificity).

2.6. Saturation kinetics

Saturation kinetics of [^{3}H] biotin in the presence of varying concentrations (0.1–100 μM) of unlabeled biotin and prodrugs is determined on MDCK-MDR1 cells according to a previously published method (Anand et al., 2003b; Suresh et al., 2010). Briefly, various concentrations of the prodrugs are prepared in DPBS spiked with 0.5 μCi/ml of [^{3}H] biotin. The medium is aspirated and cells are rinsed thrice with DPBS. Uptake is initiated by adding 1 ml of drug solution. After 30 min, solutions are removed and uptake process is terminated with ice cold stop solution. Cells are lysed overnight with 1 ml of lysis solution (0.1%, w/v, Triton X-100 in 0.3 N sodium hydroxide) at room temperature. Subsequently, aliquots (500 μl) of cell lysate are withdrawn from each well and transferred to scintillation vials containing 3 ml scintillation cocktail (Fisher Scientific). Samples are then analyzed by liquid scintillation spectrophotometer with a Beckman scintillation counter (Model LS-6500, Beckman Instruments, Inc.). The rate of uptake is normalized to protein content of each well. The data is fitted to Michaelis–Menten equation to calculate the apparent affinity constant (K_m) and maximum velocity of uptake (V_{max}).

2.7. Dose dependent inhibition studies

Dose dependent inhibition studies of [^{3}H] biotin on MDCK-MDR1 cells are studied in the presence of various concentrations (0.1–100 μM) of unlabeled biotin and biotinylated prodrugs. The study is performed using the similar procedure described earlier in Section 2.6. The data is fitted to calculate the half maximal inhibitory concentration (IC_{50}) following a previously published method (Kwatra et al., 2010; Vadlapatla et al., 2011).

2.8. Permeability studies

Permeability of [^{3}H] biotin (0.5 μCi/ml) across monolayers of MDCK-MDR1 cells in absence and presence of 50 μM concentration of unlabeled biotin and prodrugs B-ACV, B-R-ACV, and B-12HS-ACV has been determined. This study is performed to ascertain whether these prodrugs are recognized by SMVT and thus share the same transporter for their translocation. Cells were grown on transwell inserts in 12-well plates. Prior to experimentation, medium is removed and monolayers are washed 3 times with DPBS pH 7.4 and equilibrated for 30 min at 37 °C. Volumes of apical and basal chambers are 0.5 and 1.5 ml, respectively. Test solutions containing [^{3}H] biotin alone and with respective prodrugs are added on donor side and the receiving chamber contains only DPBS. Transport experiment is conducted over a period of 3 h. Samples (200 μl) are withdrawn from the receiving chamber at predetermined time points (0, 15, 30, 45, 60, 90, 120, 150 and 180 min) and replaced with equal volume of DPBS to maintain sink conditions. Samples are transferred to scintillation vials containing 3 ml of scintillation fluid (Fisher Scientific), and radioactivity is analyzed with a Beckman Scintillation Coulter (Model LS-6500, Beckman Instruments, Inc.).

2.9. Cytotoxicity measurements

Cytotoxicity assay is carried out with Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI). MDCK-MDR1 and Caco-2 cells are grown on 96 well plates. Sterile drug solutions (100 μM) are made in the culture medium using 0.22 μm nylon sterile membrane filters. Aliquots of prodrugs in culture medium (100 μl) have been added to each well and incubated for 48 h. Proliferation of the cells in the presence of ACV and its prodrugs is compared with a positive control (medium without drug) and a negative control (medium without cells). After 48 h of incubation, 20 μl dye solution is added to each well and incubated for 3 h allowing the dye to react. The amount of farmazan formed is measured with a 96-well micro titer plate reader (SpectraFluor Plus, Tecan, Maennedorf, Switzerland), absorbance set at 490 nm wavelength. As the amount of farmazan formed is directly

proportional to the viability of the cells, the toxicity of these prodrugs can be estimated.

2.10. Data analysis

All the non-radioactive samples were analyzed with LC–MS/MS. A fast and sensitive LC–MS/MS method has been developed in multiple reaction monitoring (MRM) with electrospray (ES) positive ionization mode for detection of ACV and targeted lipid prodrugs. QTRAP® LC/MS/MS mass spectrometer (API 3200, Applied Biosystems/MDS Sciex, Foster City, CA, USA) is employed to analyze samples from non-radioactive cellular accumulation and inhibition studies. Chromatographic separation is achieved on XTerra® RP8 Column, 5 μm, 4.6 mm × 50 mm (Waters Corporation, Milford, MA) with an isocratic mobile phase. The mobile phase consists of 70% acetonitrile, 30% water and 0.1% formic acid which are pumped at a flow rate of 0.2 ml/min. Precursor ions of the analytes as well as internal standard are determined from spectra obtained during the infusion of standard drug/prodrug solutions with an infusion pump connected directly to the ESI source. Each of these precursor ions is subjected to collision-induced dissociation to determine their respective product ions. MRM transitions at m/z $[M+H]^+$ generated were 226.4/152.2 for ACV, 452.3/301.3 for B-ACV, 506.3/488.6 for R-ACV, 732.3/257.4 for B-R-ACV, 507.34/488.5 for 12HS-ACV, 735.6/257.3 for B-12HS-ACV and 256/152 for GCV. Peak areas for all components are automatically integrated by Analyst™ software and peak-area ratios (area of analytes to area of internal standard) are plotted against concentration by weighted linear regression (1/concentration). The analytical data resulted from prodrugs with MRM method shows a significant linearity. This method generates rapid and reproducible results.

Stocks and stock dilutions of ACV and respective prodrugs are prepared similarly following a previously published procedure (Earla et al., 2010). Samples are extracted by liquid-liquid extraction method. Ganciclovir (GCV) is used as an internal standard to ensure reproducibility and reliability of the method. Samples are thawed at room temperature. Two hundred microliter sample along with 20 μl of GCV (5 μg/ml) is extracted with 1 ml of organic solvent containing 2:3 ratios of isopropanol (IPA) and dichloromethane (DCM). The samples are vortexed for approximately 2 min and centrifuged at 12,000 × g for 15 min at 4 °C. Organic layer (850 μl) is transferred into eppendorf tubes and evaporated to dryness under speed vacuum with a Speedvac (SAVANT Instruments, Inc., Holbrook, NY). The residue is then reconstituted in 100 μl of mobile phase, vortexed for 30 s and transferred into pre-labeled vials with silanized inserts. Subsequently, 15 μl of the resulting solution is injected onto LC–MS/MS. Appropriate calibration standards of ACV and its novel prodrugs are prepared by spiking known analyte concentrations to blank cell homogenate obtained from cultured cells following similar procedure. A calibration curve is generated using calibration standards.

[^{3}H] biotin accumulated inside the cell monolayers in the presence of various concentrations of prodrugs are calculated according to Eq. (1).

$$C_{\text{sample}} = \frac{\text{CPM}_{\text{sample}}}{\text{CPM}_{\text{donor}}} \times C_{\text{donor}} \quad (1)$$

$\text{CPM}_{\text{sample}}$ and $\text{CPM}_{\text{donor}}$ denote average values of Counts per Minute (CPM) counts of sample and donor (n = 4) respectively; C_{donor} represents the concentration of donor used and C_{sample} represents the concentration of sample.

The saturation kinetics of biotin uptake is calculated by a classic Michaelis–Menten equation (Eq. (2)).

$$V = \frac{V_{\max} \times S}{K_m + S} \quad (2)$$

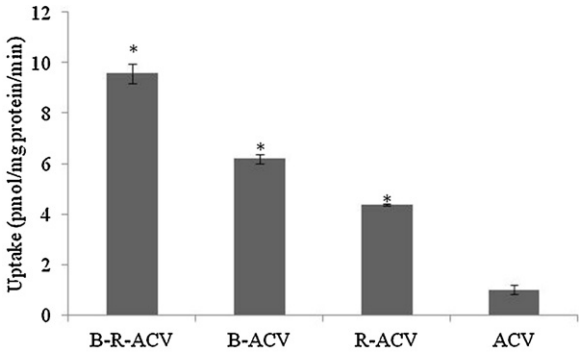

Fig. 2. Cellular accumulation of B-R-ACV, B-ACV, R-ACV and ACV on MDCK-MDR1 cells. Values represent mean ± standard deviation (n = 4) of three independent experiments. A P-value of less than 0.05 was considered to be statistically significant and denoted by asterisk (*).

V is the total rate of uptake, $V_{\max}$ is the maximum uptake rate for the carrier-mediated process and K_m is the Michaelis–Menten constant. Data is fitted to Eq. (2) using nonlinear least squares regression analysis program (KaleidaGraph 3.5).

For dose dependent inhibition studies, the inhibitory effect of [^{3}H] biotin by unlabeled biotin and biotinylated prodrugs is described by Eq. (3).

$$Y = \min + \frac{\max - \min}{1 + 10^{(\text{Log IC}_{50} - X)}} \quad (3)$$

X represents the logarithm of the concentration used; Y is the cellular accumulation of [^{3}H] biotin. Data is fitted to Eq. (3) with a transformed nonlinear regression curve analysis program (GraphPad Prism Version 4.0; GraphPad Software, Inc., San Diego, CA).

2.11. Statistical analysis

All the experiments are conducted at least in quadruplicate (n = 4), and the results were expressed as mean ± standard deviation (SD). Statistical comparison of mean values is performed with Student's t test. A P-value of less than 0.05 is considered to be statistically significant.

3. Results

3.1. Synthesis

The synthetic schemes are provided in Fig. 1A. The percent yield, mass and NMR (both ^{1}H NMR and ^{13}C NMR) spectra for all the synthesized prodrugs are given in Fig. 1B.

3.2. Cellular accumulation studies

Cellular accumulation of B-R-ACV, B-ACV, R-ACV and ACV is performed on MDCK-MDR1 cell monolayers. The results showed 9.5 times increase in the uptake of B-R-ACV compared to parent drug, ACV. B-ACV and R-ACV showed 6 times and 4 times increase in the uptake, respectively (Fig. 2). With these results, B-12HS-ACV was included along with other prodrugs for cellular accumulation studies on human intestinal Caco-2 cells following similar procedure. Compared to ACV, the uptake of B-R-ACV and B-12HS-ACV increased by 10 and 8.3 times respectively, whereas the uptake of B-ACV, R-ACV and 12HS-ACV was higher by 3.5, 1.4 and 1.3 times respectively (Fig. 3).

3.3. Inhibition study (substrate specificity)

To investigate the involvement of SMVT, the substrate specificity of the transporter is examined by inhibiting the uptake of

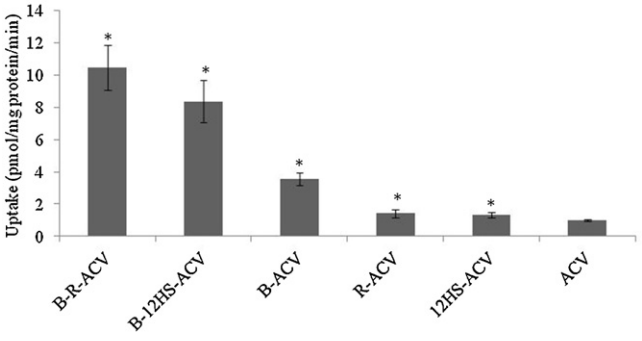

Fig. 3. Cellular accumulation of B-R-ACV, B-12HS-ACV, B-ACV, R-ACV, 12HS-ACV and ACV on Caco-2 cells. Values represent mean ± standard deviation ($n = 4$) of three independent experiments. A P-value of less than 0.05 was considered to be statistically significant and denoted by asterisk (*).

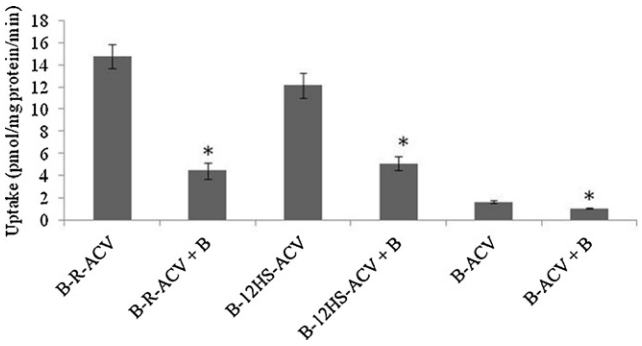

Fig. 4. Cellular accumulation of B-R-ACV, B-12HS-ACV and B-ACV in the presence of excess biotin on MDCK-MDR1 cells. Values represent mean ± standard deviation ($n = 4$) of three independent experiments. A P-value of less than 0.05 was considered to be statistically significant and denoted by asterisk (*).

B-R-ACV, B-12HS-ACV and B-ACV in presence of biotin on MDCK-MDR1 cells. The results show that the uptake is significantly lower in the presence of unlabeled biotin (Fig. 4). This result indicates that SMVT transporter may be involved in the cellular uptake of B-R-ACV, B-12HS-ACV and B-ACV.

3.4. Saturation kinetics

Uptake of [^{3}H] biotin in MDCK-MDR1 cells is studied to determine the saturation kinetics using varying concentrations (0.1–100 μM) of biotin, B-ACV, B-R-ACV and B-12HS-ACV, respectively. The results from [^{3}H] biotin uptake as a function of concentration of biotinylated prodrugs suggest a saturable component with a significant change in apparent affinity constant (K_m) (Fig. 5). Transformation of the data from the concentration dependent uptake resulted in a Lineweaver–Burk plot ($R^2 = 0.99$). The concentration dependent uptake kinetics of [^{3}H] biotin in the presence of all the biotinylated prodrugs denotes a single, saturable carrier model.

3.5. Dose-dependent inhibition of [^{3}H] biotin uptake

Dose dependent inhibition of [^{3}H] biotin uptake in MDCK-MDR1 cells has been studied with varying concentrations (0.1–100 μM) of biotin, B-ACV, B-R-ACV and B-12HS-ACV, respectively. IC_{50} values of B-R-ACV and B-12HS-ACV from the dose–response curves are calculated to be 8.04 ± 0.07 μM and 8.17 ± 0.09 μM, respectively. These values appear to be lower relative to B-ACV (14.84 ± 0.10 μM). IC_{50} value of biotin used as control is found to be 2.93 ± 0.06 μM (Fig. 6A–D).

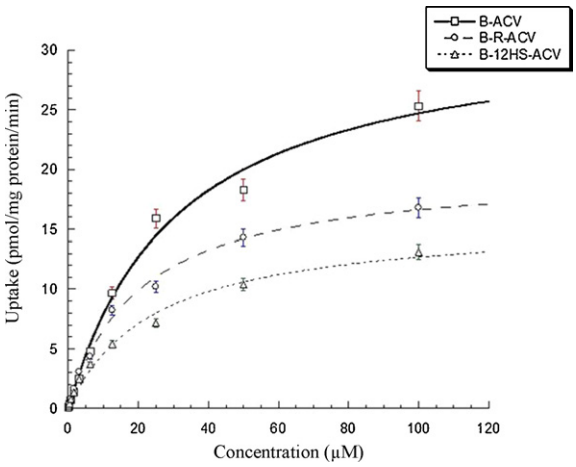

Fig. 5. Saturation kinetics across MDCK-MDR1 cells in the presence of B-R-ACV, B-12HS-ACV and B-ACV. Values represent mean ± standard deviation ($n = 4$) of three independent experiments.

3.6. Permeability studies

Transport of [^{3}H] biotin (0.5 μCi/ml) across MDCK-MDR1 monolayers was assessed. From the transport data, cumulative amount transported is plotted against time. Unlabeled biotin and all the biotinylated ACV prodrugs appears to inhibit the transport of [^{3}H] biotin, a substrate extensively studied for its translocation by SMVT (Fig. 7A). Biotinylated lipid prodrugs of ACV (B-R-ACV and B-12HS-ACV) exhibited higher inhibition in [^{3}H] biotin transport than that of B-ACV suggesting the synergistic involvement of SMVT transporter and the lipid moiety in mediating cellular permeation across MDCK-MDR1. Permeabilities of [^{3}H] biotin are 4.74×10^{-6} cm/s in comparison to 0.57×10^{-6} cm/s, 2.96×10^{-6} cm/s, 1.90×10^{-6} cm/s, 2.10×10^{-6} cm/s in presence of 50 μM concentration of unlabeled biotin and prodrugs B-ACV, B-R-ACV, and B-12HS-ACV, respectively (Fig. 7B).

3.7. Cytotoxicity assay

Cytotoxicity assay was performed on MDCK-MDR1 and Caco-2 cell monolayers for a period of 48 h to evaluate the cytotoxic effect of all the prodrugs studied. Neither ACV nor its targeted lipid prodrugs demonstrate any cytotoxic effect at the examined concentration (100 μM). The results from this assay clearly suggest that all the targeted and non-targeted lipid prodrugs studied may be comparatively less cytotoxic with respect to ACV (Fig. 8).

4. Discussion

DNA, RNA and other nucleotide-based therapeutic agents are highly hydrophilic and encounter resistance in crossing lipophilic cellular membrane. Despite advances in drug delivery technology, there still remains a need to develop newer technologies, especially for hydrophilic nucleotide-based therapeutic agents such as ACV. Therefore, we have developed novel drug conjugates in which therapeutic agents are linked to a substrate for a membrane transporter/receptor via a lipophilic raft. In this unique combination, we hypothesize that the lipid raft facilitates interaction of prodrug with cell membrane probably assisting in docking of the targeted ligand into the binding domain of transporter protein. The net effect is rapid translocation of the cargo across cell membrane. The synthesis has been carried out by conjugating the compounds via

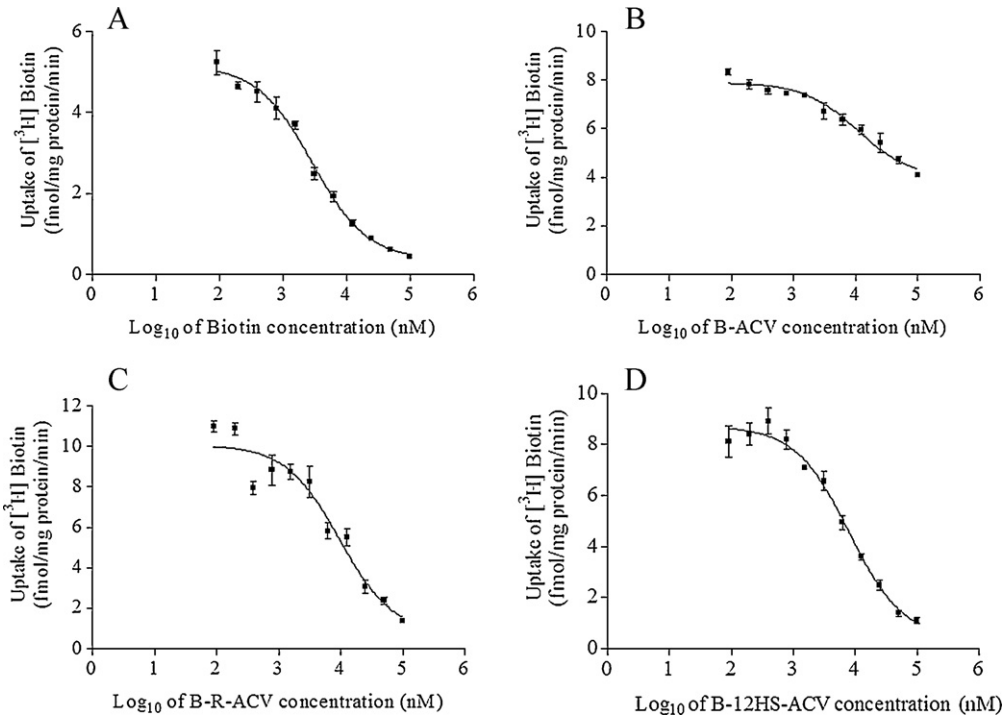

Fig. 6. Dose dependent inhibition of [^{3}H] biotin uptake in MDCK-MDR1 cells in the presence of varying concentrations (0.1–100 μM) of (A) biotin, (B) B-ACV, (C) B-R-ACV and (D) B-12HS-ACV. Values represent mean ± standard deviation (n = 4) of three independent experiments.

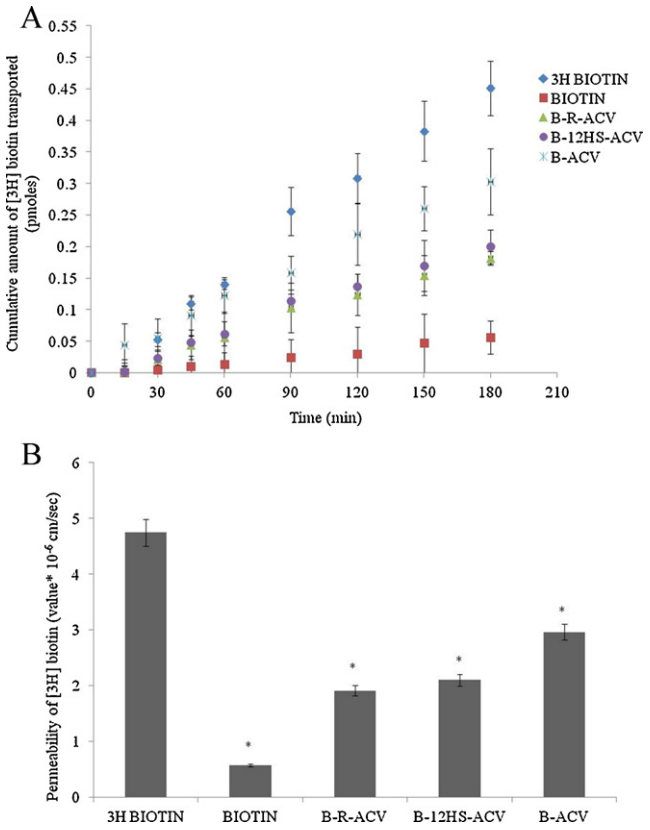

Fig. 7. (A) Transepithelial transport of [^{3}H] biotin in MDCK-MDR1 cells in the absence and presence of 50 μM unlabeled biotin, B-ACV, B-R-ACV, and B-12HS-ACV. (B) Comparison of permeabilities (cm/s) of [^{3}H] biotin alone, in the presence of 50 μM unlabeled biotin, B-ACV, B-R-ACV, and B-12HS-ACV on MDCK-MDR1 cells. Data represents mean ± standard deviation (n = 4–6) of four independent experiments. A P-value of less than 0.05 was considered to be statistically significant and denoted by asterisk (*).

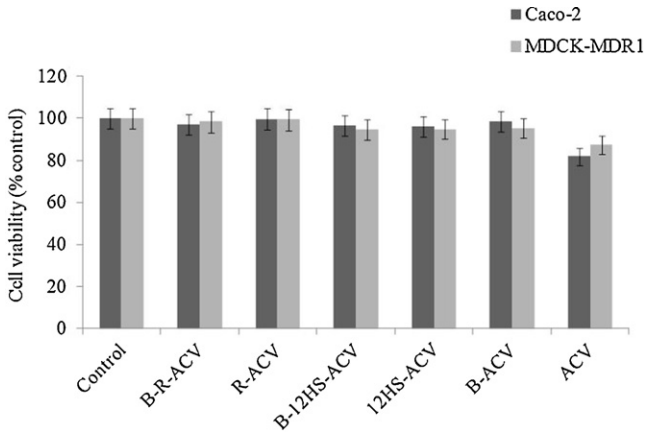

Fig. 8. Cytotoxicity assay in the presence of B-R-ACV, R-ACV, B-12HS-ACV, 12HS-ACV, B-ACV and ACV on Caco-2 and MDCK-MDR1 cells for 48 h. Data represents mean percentage of viable cells ± standard deviation (n = 4) of three independent experiments.

esterification reactions. NMR data confirms the structure of all the prodrugs synthesized (Fig. 1A and B).

To test the hypothesis that SMVT can be a target for drug delivery, cellular accumulation studies have been carried out in two different cell lines (MDCK-MDR1 and Caco-2). The results are consistent in both the cell lines which demonstrate significantly higher cellular accumulation of biotinylated lipid prodrugs (B-R-ACV and B-12HS-ACV) relative to only biotinylated (B-ACV) or only lipid conjugated compounds (R-ACV and 12HS-ACV). Such rise in intracellular drug accumulation may presumably be due to a combined effect of higher transcellular diffusion due to enhanced lipophilicity and significant carrier-mediated transport by SMVT (Figs. 2 and 3).

MDCK-MDR1 cells have been selected for inhibition, saturation kinetics and dose dependent studies because it has been shown as

an alternative to Caco-2 cell line for high throughput screening in drug discovery (Tang and Borchardt, 2002; Tang et al., 2002, 2004). Specific interaction of B-R-ACV, B-12HS-ACV and B-ACV with SMVT (substrate specificity) has been examined by inhibiting the cellular uptake of these prodrugs across MDCK-MDR1 cell monolayers in the presence of excess biotin. Accumulation of B-R-ACV, B-12HS-ACV and B-ACV are significantly lowered supporting the hypothesis that these compounds are transported primarily by SMVT (Fig. 4).

The results from concentration dependent (saturation kinetics) studies reveal lower K_m values of B-R-ACV ($20.25 \pm 1.74\ \mu M$) and B-12HS-ACV ($23.99 \pm 3.20\ \mu M$) relative to B-ACV ($30.90 \pm 4.19\ \mu M$) demonstrating an improved affinity of targeted lipid prodrugs towards SMVT (Fig. 5). The apparent affinity constant (K_m) values of all the biotinylated prodrugs are found to be slightly higher relative to biotin ($12.25 \pm 0.79\ \mu M$) indicating that the prodrugs may not have similar affinity for SMVT relative to biotin itself. Lower K_m values of B-R-ACV and B-12HS-ACV may be attributed to the presence of lipid raft between the targeting moiety and the drug, which may assist in enhancing the binding affinity of these prodrugs to SMVT. The data is then transformed into Lineweaver–Burk plots to determine the nature of the inhibition process i.e. competitive or non-competitive. The transformations suggest the inhibition to be competitive, revealing that these prodrugs may share a common site for biotin in SMVT structure (data not shown). This data further confirms the hypothesis that the lipoidal linker is further enhancing the affinity of substrate toward SMVT.

The half-maximal inhibitory concentration (IC_{50}) for inhibition of [^{3}H] biotin uptake by the prodrug is a further indication of the enhanced affinity of the test compound for the SMVT transporter. A comparison of IC_{50} values clearly suggests that biotin exhibits much higher affinity toward SMVT relative to biotinylated prodrugs. The targeted lipid prodrugs B-R-ACV and B-12HS-ACV appear to have comparatively lower IC_{50} values of $8.04 \pm 0.07\ \mu M$ and $8.17 \pm 0.09\ \mu M$, respectively relative to biotinylated prodrug B-ACV (IC_{50} value of $14.84 \pm 0.10\ \mu M$) (Fig. 6). These values suggest that the presence of a lipid raft in targeted lipid prodrugs further aids in enhancing the inhibitory potential of the compound compared to non-lipidated biotin conjugated prodrug. The results from this study strongly correlates to the one obtained from the saturation kinetic studies.

[^{3}H] biotin transport results are consistent with a previous report published from our laboratory using MDCK-MDR1 cells (Luo et al., 2006). [^{3}H] biotin permeability is assessed in the presence of biotin and various prodrugs (Fig. 7A). Inhibition of [^{3}H] biotin transport probably indicates an interaction of these newly synthesized biotinylated prodrugs with SMVT. A significantly elevated enhancement in the inhibition of [^{3}H] biotin permeability by B-R-ACV and B-12HS-ACV compared to B-ACV may be attributed to our current approach which combines both lipid and transporter targeted delivery to generate a synergistic transport effect (Fig. 7B). The results from cytotoxicity assay showed that all of these prodrugs are safe and exhibit much lower cytotoxicity relative to ACV itself (Fig. 8).

In conclusion, results from this study clearly demonstrate that targeted lipid prodrugs of ACV exhibit higher affinity towards SMVT. Cellular accumulation of these prodrugs is mainly mediated by SMVT as biotin uptake can be significantly inhibited. The lipid raft facilitates enhanced interaction of prodrug with membrane proteins probably assisting docking of the targeted ligand into the binding domain of transporter/receptor protein. The net effect is rapid translocation of the cargo across cell membrane. This novel technology may also allow for enhanced plasma membrane uptake of various hydrophilic therapeutic agents such as nucleosides, nucleotides, oligonucleotides or antisense oligonucleotides and peptides.

Acknowledgment

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