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Targeting SVCT for enhanced drug absorption: Synthesis and *in vitro* evaluation of a novel vitamin C conjugated prodrug of saquinavir

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ABSTRACT

In order to improve oral absorption, a novel prodrug of saquinavir (Saq), ascorbyl-succinic-saquinavir (AA-Su-Saq) targeting sodium dependent vitamin C transporter (SVCT) was synthesized and evaluated. Aqueous solubility, stability and cytotoxicity were determined. Affinity of AA-Su-Saq towards efflux pump P-glycoprotein (P-gp) and recognition of AA-Su-Saq by SVCT were studied. Transepithelial permeability across polarized MDCK-MDR1 and Caco-2 cells were determined. Metabolic stability of AA-Su-Saq in rat liver microsomes was investigated. AA-Su-Saq appears to be fairly stable in both DPBS and Caco-2 cells with half lives of 9.65 and 5.73 h, respectively. Uptake of [^{3}H]Saquinavir accelerated by 2.7 and 1.9 fold in the presence of 50 μM Saq and AA-Su-Saq in MDCK-MDR1 cells. Cellular accumulation of [^{14}C]AA diminished by about 50–70% relative to control in the presence of 200 μM AA-Su-Saq in MDCK-MDR1 and Caco-2 cells. Uptake of AA-Su-Saq was lowered by 27% and 34% in the presence of 5 mM AA in MDCK-MDR1 and Caco-2 cells, respectively. Absorptive permeability of AA-Su-Saq was elevated about 4-5 fold and efflux index reduced by about 13-15 fold across the polarized MDCK-MDR1 and Caco-2 cells. Absorptive permeability of AA-Su-Saq decreased 44% in the presence of 5 mM AA across MDCK-MDR1 cells. AA-Su-Saq was devoid of cytotoxicity over the concentration range studied. AA-Su-Saq significantly enhanced the metabolic stability but lowered the affinity towards CYP3A4. In conclusion, prodrug modification of Saq through conjugation to AA via a linker significantly raised the absorptive permeability and metabolic stability. Such modification also caused significant evading of P-gp mediated efflux and CYP3A4 mediated metabolism. SVCT targeted prodrug approach can be an attractive strategy to enhance the oral absorption and systemic bioavailability of anti-HIV protease inhibitors.

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1. Introduction

Saquinavir (Saq) is the first protease inhibitor (PI) approved by FDA for the treatment of HIV infected patients. Although Saq possesses high anti-HIV potency, therapeutic efficacy of this compound is very limited due to several unfavorable physicochemical and pharmacokinetic properties such as low aqueous solubility, low absorptive permeability and rapid biotransformation into inactive metabolites. Currently there are two marketed formulations including a hard-gel capsule of saquinavir mesylate (Invirase®), and a soft-gel capsule of saquinavir (Fortovase®). However, systemic bioavailability of these formulations falls in the very low range of 4–12% (Figgitt and Plosker, 2000). Several strategies have been employed to improve oral absorption and bioavailability of protease inhibitors (PIs). Earlier approaches include functional inhibition of P-gp and/or CYP3A4 by co-administrating P-gp and/or CYP3A4 modulators (Varma et al., 2003; Katragadda et al., 2005) and modification of the physicochemical properties of the permeants (Wire et al., 2006). Although inhibition of P-gp/CYP3A4 activities may lead to higher bioavailability, these approaches are largely hindered by the side effects such as perturbing normal physiological roles of these proteins and increasing cytotoxicity due to the high doses required to inhibit the functions of P-gp/CYP3A4. Recently, transporter targeted prodrug approach has been developed as an attractive strategy to enhance drug delivery. Targeted prodrug design approaches have been discussed in a few review articles (Han and Amidon, 2000; Yang et al., 2001; Majumdar et al., 2004; Rautio et al., 2008; Palombo et al., 2009). Apart from classical prodrugs, which modulate hydrophilicity/lipophilicity properties of the active ingredient by covalently linking hydrophilic/lipophilic pro-moieties, transporter targeted prodrugs are designed to enhance drug absorption by conjugating the parent drug molecules with specific substrates of specific membrane nutrient transporters. Such modified drug agents can be recognized by the influx transporters and exhibit reduced affinity towards efflux and metabolizing proteins. A number of nutrient

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transporters expressed on various epithelial and endothelial cellular membrane surfaces have been explored for the targeted drug delivery of PIs (Rouquayrol et al., 2002; Jain et al., 2005; Luo et al., 2006). These nutrient transporters are responsible for the influx of various nutrients i.e. amino acids, peptides, glucose and vitamins. Such transporter targeted prodrugs can be ferried by the nutrient transporters and translocated across epithelial membrane. After the conjugates permeate across plasma membrane, these compounds are cleaved by hydrolytic enzymes in either systemic circulation or target tissues. Then free active parent drug is regenerated. Importantly, the released nutrient pro-moieties are noncytotoxic.

The aim of the present work is to explore the application of sodium dependent vitamin C transporter (SVCT) for enhanced delivery of PIs. Vitamin C, also known as ascorbic acid (AA), is an essential water-soluble vitamin for mammalian cell growth, function and recovery. It serves as an antioxidant agent and plays an essential role in the defense against free radicals (Rose, 1988; Wilson, 2005). Recent studies demonstrated that AA and analogs may possess antiviral (Harakeh et al., 1990; Furuya et al., 2008) and anti-tumor (Kong et al., 2009) activities. Two isoforms of sodium-dependent vitamin C transporters, SVCT1 and SVCT2, have been cloned from human and rat DNA libraries (Daruwala et al., 1999; Tsukaguchi et al., 1999; Wang et al., 1999). Both SVCT carriers have similar functions and can mediate the transport of AA with high affinity. SVCT1 is mainly expressed in epithelial cells of kidney, intestine and liver, whereas SVCT2 is widely distributed in brain, eye and other organs (Tsukaguchi et al., 1999). Residence of SVCT1 in apical membrane of polarized cells has been reported for both Caco-2 and MDCK cells by functional and confocal imaging studies (Maulen et al., 2003; Boyer et al., 2005; Luo et al., 2008).

Recently, SVCT2-mediated delivery of neurotropic agents to the central nervous system (CNS) was reported (Manfredini et al., 2002, 2004; Dalpiaz et al., 2004, 2005). Several AA or AA derivative (BrAA, AA-NH) conjugated prodrugs of nipecotic (Nipec), kynurenic (Kynur) and diclofenamic (Diclo) acids were evaluated. It appears that SVCT2 was involved in both cellular accumulation of BrAA-Nipec into human retinal pigment epithelium (HRPE) cells and the transport of AA-Nipec to mouse brain eliciting anticonvulsant effect.

We have previously reported the expression of SVCTs in polarized MDCK-MDR1 cells (Luo et al., 2008). SVCT1 was dominantly expressed on the apical side of the cellular membrane. The current work is to explore this transport system for the enhanced delivery of Saq. A novel vitamin C conjugated prodrug (AA-Su-Saq) was designed to circumvent P-gp mediated efflux and to increase metabolic stability of Saq. Utilizing both MDCK-MDR1 and Caco-2 cell lines, uptake studies were conducted to test whether the prodrug can bypass the efflux protein P-gp and be recognizable by the influx transporter SVCT. Transepithelial transport studies were carried out to determine the permeability of the prodrug across polarized epithelial cells. Stabilities of the prodrug in both DPBS buffer and Caco-2 cell homogenates were evaluated. Rat liver microsomes were employed to investigate the metabolism kinetics of the prodrug. Cytotoxicity of the prodrug was also evaluated with MTT assay.

2. Experimental

2.1. Materials

[^{14}C]Aascorbic acid ([^{14}C]AA, specific activity 8.5 mCi/mmol) and [^{3}H]Saquinavir ([^{3}H]Saq, specific activity 1.0 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). Saquinavir mesylate was generously supplied by Hoffmann-La Roche (Nutley, NJ). The prodrug used in this study was synthesized in our laboratory. L-Ascorbic acid (AA) and all other chemicals were obtained from Sigma Chemical Company (St. Louis, MO). Rat liver microsomes were obtained from XenoTech, LLC (Lenexa, KS).

MDCK-MDR1 cells were obtained as a gift from P. Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands). The growth medium Dulbecco modified Eagle medium (DMEM) and nonessential amino acids (NEAA) were obtained from Gibco (Invitrogen, Grand Island, NY). Penicillin, streptomycin, sodium bicarbonate and HEPES were purchased from Sigma Chemical Company. Calf serum, fetal bovine serum (FBS) and trypsin/EDTA solutions were obtained from JRH Bioscience (Lenexa, KS).

Buffer used in the uptake studies is Dulbecco modified phosphate buffer saline (DPBS), containing 130 mM NaCl, 7.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.03 mM KCl, 5.3 mM glucose and 25 mM Hepes. These chemicals, of analytical grade, were 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).

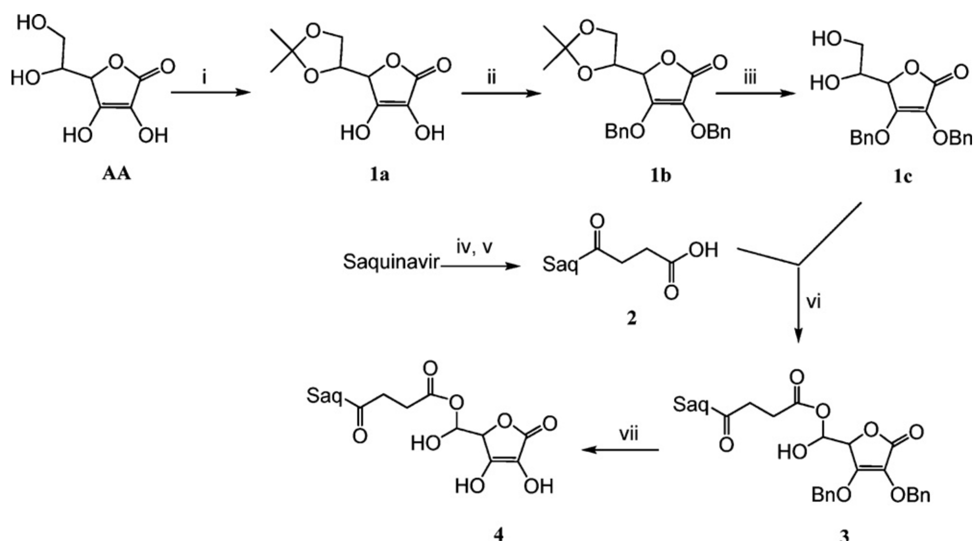
2.2. Synthesis of ascorbic succinic saquinavir (AA-Su-Saq)

The novel prodrug of Saq (AA-Su-Saq) was synthesized in our laboratory. The overall synthetic pathway is shown in Scheme 1. Free C-2 and C-3 hydroxyl groups are critical for AA to be recognized by SVCT. In order to selectively react with the primary hydroxyl group at position C-6, the OH groups at positions C-2 and C-3 need to be protected before the conjugation. As both AA and Saq have only hydroxyl groups, a spacer with two carboxyl groups is necessary to link them together. Overall, AA-Su-Saq was formed by conjugating Saq with AA via a linker succinic acid (Su). All chemicals were obtained from commercial suppliers and were of reagent grade. The reactions were performed under anhydrous nitrogen atmosphere unless otherwise indicated.

The intermediate O^2, O^3-dibenzyl-AA (**1c**) was synthesized following the procedures reported in the literature (Von Dallacker and Sanders, 1985). The overall yield from AA to O^2, O^3- dibenzyl-AA (**1c**) was 50%.

Saq was linked with mono-*tert*-butyl succinic acid before conjugating with O^2,O^3- dibenzyl-AA (**1c**). A mixture of mono-*tert*-butyl succinic acid (0.45 g, 2.6 mmol) and N,N-dicyclohexylcarbodimide (DCC, 0.54 g, 2.6 mmol) in 20 mL CH_2Cl_2 (mixture A) was stirred for 1 h at 0 °C under N_2 atmosphere. Another mixture of saquinavir mesylate (Saq, 1 g, 1.3 mmol), triethylamine (TEA, a few drops) and dimethylaminopyridine (DMAP, 0.32 g, 2.6 mmol) in 10 mL CH_2Cl_2 (mixture B) was stirred for about 10 min. Then, mixture B was added dropwise into mixture A. The reaction mixture was continually stirred for 24 h at room temperature (RT) under N_2 atmosphere. The reaction was monitored with TLC and mass spectroscopy (MS). After the reaction was completed, the precipitate (DCU) was filtered and then solvent was evaporated, the residue purified using silica gel chromatography with dichloromethane (DCM, CH_2Cl_2) and methanol (97:3) as eluent. The removal of the protecting *tert*-butyl group was achieved by reacting with trifluroacetic acid (TFA) and DCM (4:1) at 0 °C for 5 h. After evaporating DCM and excess TFA, the crude product was purified by precipitating with cold diethyl ether and dried in Speed Vac (SPD101B, Savant Instruments Inc., Holbrook, NY) to a constant weight. The final product saquinavir succinate (Saq-Su, compound **2**) was obtained as white powder with a yield of 80%.

Compound **2** was conjugated with protected AA (compound **1c**) in the presence of DCC and DMAP according to above described procedures. A mixture of Saq-Su (**1b**, 0.771 g, 1 mmol) and DCC



Scheme 1. Synthesis of ascorbic-succinic-saquinavir (AA-Su-Saq). *Reagents and conditions*: (i) acetyl chloride, acetone, 4 h, RT. (ii) K_2CO_3, benzyl chloride, DMF, 60 °C, 8 h. (iii) 50% acetic acid, methanol, 100 °C, 5 h. (iv) DCC, DMAP, α-(*tert*-butyl) succinic acid, CH_2Cl_2, 24 h. (v) TFA, RT, 2 h. (vi) DCC, DMAP, CH_2Cl_2, 24 h. (vii) H_2, 10% Pd/C, THF, 24 h.

(0.412 g, 2 mmol) in 20 mL CH_2Cl_2 (mixture C) was stirred for 1 h at 0 °C under N_2 atmosphere. A mixture of O^2,O^3- dibenzyl-AA (**1c**, 0.427 g, 1.2 mmol) and DMAP (0.244 g, 2 mmol) in 10 mL CH_2Cl_2 (mixture D) was stirred at RT for 10 min. Mixture D was added dropwise to mixture C. The reaction was continually stirred at RT for 24 h. The progress was monitored with TLC and MS until the starting material **1b** disappeared. After evaporating the solvent under vacuo, the crude was purified by silica gel chromatography in DCM and methanol (9:1). Compound **3** was obtained as white powder with a yield of 60%.

The final product, ascorbic-succinic-saquinavir (AA-Su-Saq, compound **4**) was obtained after removing the benzyl protecting groups of compound **3** by hydrogenation catalyzed by Pd/C. A mixture of compound **3** (0.554 g, 0.5 mmol) and 10% Pd/C (0.11 g) in 50 mL tetrahydrofuran (THF) was shaken overnight with addition of hydrogen (H_2, 30 psi) by Parr shaker type hydrogenator (Parr Instrument Company, Moline, IL). The reaction was monitored with TLC and MS until it was completed. After filtration and evaporation, the crude was purified by silica gel chromatography in DCM and methanol (9:1 then 4:1) to obtain the final product AA-Su-Saq (compound **4**) with a yield of 70%.

The structure and purity of the reaction intermediates (O^2,O^3-dibenzyl-AA and Saq-Su) and the final product (AA-Su-Saq) were confirmed by ^{1}HNMR and mass spectroscopy (MS). ^{1}HNMR was carried out with a Varian-400 MHz NMR spectrometer.

O^2,O^3-Dibenzyl-AA (compound **1c**): MS ((*m/z*): 357.2; Calculated ($C_{20}H_{20}O_6$): 356.1.

^{1}HNMR (DMSO-d_6): δ 7.38 (m, 10 H), 5.15–5.24 (m, 3H), 4.89–4.96 (m, 4H), 3.71 (d, 1H), 3.45 (m, 2H).

Saq-Su (compound **2**): MS ((*m/z*): 771.5; Calculated ($C_{42}H_{54}O_6$): 770.4.

^{1}HNMR (DMSO-d_6): δ 12.3 (1H, bd), 9.59 (1H, bd), 8.82 (d, 1H), 8.60 (d, 1H), 8.32 (bd, 1H), 8.10-8.17 (m, 4H), 7.91 (m, 1H), 7.75 (m, 1H), 7.48 (s, 1H), 7.16 (m, 3H), 7.04 (d, 1H), 6.98 (s, 1H), 5.37 (1H, bd), 4.76 (d, 1H), 4.28 (s, 1H), 3.18–3.59 (m, 10H), 2.89 (s, 1H), 2.73 (s, 1H), 2.61 (m, 1H), 1.32 (s, 9H), 1.05–1.92 (m, 12H).

AA-Su-Saq (compound **4**): MS (*m/z*): 929.5; Calculated ($C_{48}H_{60}N_6O_{13}$): 928.4.

^{1}HNMR (DMSO-d_6): δ 8.84 (d, 1H), 8.59 (d, 1H), 8.31 (bd, 1H), 8.10–8.17 (m, 4H), 7.92 (m, 1H), 7.75 (m, 1H), 7.48 (m, 1H), 7.33 (m, 1H), 6.90 (m, 1H), 7.02 (m, 2H), 7.24 (m, 2H), 5.38 (m, 1H), 4.76 (m, 1H), 4.41 (m, 1H), 3.83 (m, 2H), 3.65 (m, 1H), 3.48 (m, 1H), 2.93 (m, 2H), 2.86 (m, 1H), 2.82 (d, 1H), 2.64–2.78 (m, 6H), 2.37 (m, 1H), 2.21 (d, 1H), 1.34 (s, 9H), 1.10–2.02 (m, 12H).

2.3. Cell culture

MDCK-MDR1 cells (passages 5–15) were cultured in DMEM supplemented with 10% heat inactivated (HI) calf serum (CS), 1% nonessential amino acids, 100 U/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% humidity for 3–4 days to reach 80% confluence, and then were plated at a density of 66,000 cm^{-2} in 12-well tissue culture-treated plastic plates, and incubated at 37 °C in humidified atmosphere of 5% CO_2 and 95% air grown for 6–7 days to reach confluence before the experiments. The medium was changed every other day.

Caco-2 cells (passages 26–35) were grown under similar conditions except 10% HI FBS was used instead of CS. Cells were grown for 21–23 days before initiation of an experiment.

2.4. Aqueous solubility study

Five milligram saquinavir mesylate (Saq) or prodrug (AA-Su-Saq) was added to 2 mL of distilled deionized water (DDW) in a 10 mL screw-capped glass tubes. These tubes were placed in water incubation bath at 25 °C and mechanically shaken for 24 h. After 24 h, the mixture was centrifuged at 12,500 rpm for 10 min. The supernatant was separated and filtered with 0.45 μm membrane (Nalgene syringe filter). The filtrate was diluted with DDW and the concentration was measured by LC–MS/MS.

2.5. Stability studies in DPBS buffer and Caco-2 cell homogenates

Confluent Caco-2 cells were washed three times with cold DPBS. Cells were isolated with the aid of a mechanical scraper and then suspended in proper volume of chilled DPBS and homogenized using Multipro variable speed homogenizer (DREMEL, Rachi, WI) for 5 min on an ice bath. Cell homogenates were centrifuged at 12,500 rpm, 4 °C for 10 min to remove debris. The supernatant was diluted properly to achieve a final protein concentration of 0.30 mg/mL. Protein content was determined using BioRad protein estimation kit (BioRad, Hercules, CA).

Prodrug solutions in DPBS or cell homogenates were freshly prepared. Stock solution of prodrug (10 mM) was prepared in dimethyl sulfoxide (DMSO) and used immediately. Five milliliters of DPBS or Caco-2 cell homogenates were placed in screw capped vials and allowed to equilibrate at 37 °C for 15 min before the experiments started. Prodrug solution was spiked into DPBS or cell homogenates, resulting in a final concentration of 10 μM. Vials were placed in a water bath at 37 °C and shaken at 60 rpm for 12 h. Aliquots (100 μL) were withdrawn at predetermined time points and an equal volume of ice-cold acetonitrile was added to stop the enzymatic reactions. Samples were stored at −80 °C until further analysis by LC–MS/MS. All experiments were conducted at least in triplicate.

2.6. Cytotoxicity study

Cytotoxicity of AA-Su-Saq in MDCK-WT cells was determined applying MTS assay following manufacturer's protocol (Invitrogen, Carlsbad, CA). The assay determines cell viability based on the mitochondrial conversion of a water-soluble tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolum bromide, MTT] to the water-insoluble blue formazan product. Cytotoxicity kit is supplied as a salt solution of MTT (known as MTS) with an electron coupling reagent phenazine methosulfate (PMS). Briefly, MDCK-WT cells were seeded on 96-well plates and grown overnight. Then culture medium was replaced with 100 μL of serial AA-Su-Saq prodrug solutions (10–400 μM). Cells were then incubated for 6 h at 37 °C. After the treatment was over, 20 μL MTS dye solution was added and then incubated for another 2 h at 37 °C. Finally, absorbance at 485 nm was measured on an automated microplate photometer (Biorad, Hercules, CA).

2.7. Uptake studies

2.7.1. Uptake studies with radiolabeled compounds

Uptake studies were conducted with confluent cells. The medium was removed, and cells were rinsed 3 times, 10 min each with 2 mL of DPBS at 37 °C, unless otherwise stated. In a typical uptake experiment with radiolabeled compounds, cells were incubated with 1 mL of [^{3}H]Saq (0.5 μCi/mL) or [^{14}C]AA (0.2 μCi/mL) in the absence or presence of predefined inhibitors prepared in DBPS at 37 °C for 30 min. For the uptake experiments of [^{14}C]AA, 0.5 mM dithiothreitol (DTT) was added to DPBS to prevent the oxidation of AA. 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. Then cells were left overnight in 1 mL lysis solution [0.1% (v/v) Triton X-100 in 0.3 N NaOH] at RT. Aliquots (500 μL) from each well were then transferred to scintillation vials containing 5 mL scintillation cocktail (Fisher Scientific, Fairlawn, NJ). Samples were analyzed with liquid scintillation counter (Model LS-6500, Beckman Instruments, Inc., Fullerton, CA). The rate of uptake was normalized to the protein content of each well. Amount of protein in the cell lysate was measured by a BioRad protein estimation kit (BioRad, Hercules, CA).

2.7.1.1. Inhibition of [3H]Saq uptake by Saq and AA-Su-Saq. Unlabeled Saq and AA-Su-Saq were dissolved in DMSO to generate 50 mM stock solution. Fifty micromolar solutions were made by diluting the stock solution with DPBS. Then [^{3}H]Saq was added right before an experiment. Inhibitory studies were conducted using MDCK-MDR1 cells according to the procedure described in Section 2.7.1.

2.7.1.2. Inhibition of [^{14}C]AA uptake by AA and AA-Su-Saq. Unlabeled AA and AA-Su-Saq were dissolved in DMSO to generate 100 mM stock solution. Then 200 μM solutions were prepared by diluting the stock solution with DPBS. Then [^{14}C]AA was added right before an experiment. Uptake studies were conducted using both MDCK-MDR1 and Caco-2 cells according to the procedure described in Section 2.7.1.

2.7.2. Uptake of prodrug in MDCK-MDR1 and Caco-2 cells

Cellular accumulation of AA-Su-Saq (25 μM) was challenged with the addition of unlabeled AA (5 mM) to the permeant solutions. Uptake experiments were conducted employing both MDCK-MDR1 and Caco2 cells for 60 min at 37 °C. Following the incubation, cells were washed with cold DBPS three times and then lysed by adding DPBS (0.5 mL per well of 12-well plate) followed by freezing at −80 °C for 30 min. Cells were then thawed on ice, and lysates were centrifuged at 12,500 rpm for 15 min to remove cell membrane debris. The supernatant was extracted with 600 μL water saturated ethyl acetate. The upper layer was separated and evaporated under vacuo. The samples were reconstituted in mobile phase for LC–MS/MS analysis.

2.8. Transepithelial transport studies

2.8.1. Transepithelial transport of [^{14}C]AA across Caco-2 cells

Bidirectional transepithelial transport of [^{14}C]AA in Caco-2 cells was first studied in 12-well Transwell® plates. Concentration of [^{14}C]AA added to the donor chamber was 0.2 μCi/mL (23.5 μM). Before each experiment, cell monolayers were washed 3 times with DPBS at 37 °C. Working volumes of apical (A) and basolateral (B) chambers were 0.5 and 1.5 mL, respectively. Transport was initiated by adding drug solution in the donor chamber and DPBS in the receiving chamber. Aliquots (100 μL) were withdrawn from the receiving chamber at predetermined time points over a period of 3 h and replaced with same volumes of DPBS to maintain sink conditions. Samples were analyzed with a liquid scintillation counter.

2.8.2. Transepithelial transport of AA-Su-Saq across MDCK-MDR1 and Caco-2 cells

Transepithelial transport studies of AA-Su-Saq were then conducted in both MDCK-MDR1 and Caco-2 cells following similar procedure as above. Initial concentration of AA-Su-Saq added to the donor chamber was 25 μM. Aliquots (200 μL) were withdrawn from the receiving chamber at predetermined time intervals over a period of 3 h and replaced with same volumes of DPBS. Samples were stored at −80 °C until further analysis. Samples were prepared by liquid–liquid extraction and then analyzed by LC–MS/MS.

Transepithelial A–B transport of AA-Su-Saq across MDCK-MDR1 cells was challenged with the addition of unlabeled AA (5 mM) following above procedure.

2.9. Metabolism studies

First, the stock solutions of various reagents were prepared in DDW. Stock solutions were prepared with magnesium chloride ($MgCl_2$) 1 M, glucose 6-phosphate (G-6-P) 500 mM, G-6-P dehydrogenase (G-6-P DH) 100 U/mL and NADP 100 mM. The stock solutions of drug and prodrug were prepared as 10 mM in DMSO. Then NADPH generating system was freshly prepared by adding G-6-P, G-6-P DH and NADP aqueous solutions. The diluted drug and prodrug solutions, phosphate buffer and microsomes were mixed and pre-incubated at 37 °C for 5 min. The metabolic reaction was initiated by adding NADPH generating system. The final concentrations of various reagents in individual incubate solution were: 0.30 mg/mL microsome, 5 mM $MgCl_2$, 5 mM G-6-P, 1 U/mL G-6-P DH and 1 mM NADP. For time dependent studies, the initial concentration of drug and prodrug was 2 μM and samples (100 μL) were taken at predetermined time points (0, 1, 2.5, 5, 10, 20, 40, 60, 90 and 120 min). The metabolic reaction was stopped by adding equal volume of ice-cold acetonitrile to the sample. Samples were

stored at −80 °C until further analysis. Samples were prepared by liquid–liquid extraction and analyzed with LC–MS/MS.

2.10. Sample preparation

Samples from stability, uptake, transport and metabolic studies were prepared by liquid–liquid extractions. Verapamil was used as an internal standard (IS). Organic solvents, methyl *tert*-butyl ether (MTBE) and water saturated ethyl acetate, were utilized to extract Saq and AA-Su-Saq from aqueous buffer solution, respectively. Briefly, 10 μL of IS solution was added to the aqueous solution of samples after acetonitrile was evaporated. Then, 2 times of sample volume of organic solvent was added and vortexed for 2 min to allow enough time for the drug or prodrug partition between aqueous and organic phases. After vortexing, the mixture was centrifuged at 12,500 rpm for 10 min to allow sufficient separation of the aqueous and organic layers. Then, samples of Saq with MTBE and AA-Su-Saq with ethyl acetate were placed −80 and −20 °C for 30 min, respectively. The organic layer was then separated and dried under vacuo. The residue was reconstituted in 100 μL of mobile phase. Standard solutions (in DPBS) of drug and prodrug were also extracted following the same procedures. All reconstituted samples were analyzed by LC–MS/MS. Relative extraction efficiencies were calculated.

2.11. LC–MS/MS analysis

Samples collected from stability, uptake, transport and metabolic studies were analyzed by LC–MS/MS. QTrap® LC–MS/MS mass spectrometer (Applied Biosystems/Sciex) equipped with Agilent 1100 series quaternary pump (Agilent G1311A), vacuum degasser (Agilent G1379A), and autosampler (Agilent G1367A) was employed. HPLC separation was performed on a Luna C18(2) column 100 mm × 2.0 mm, 3 μm (Phenomenex, Torrance, CA). The mobile phase was composed of 64% acetonitrile and 36% DDW including 0.1% formic acid and run at a flow rate of 0.2 mL/min. The sample (30 μL) was injected and chromatograms were collected over 5 min. Electrospray ionization (ESI) in positive mode was employed for the sample introduction. The detection was operated in multiple-reaction monitoring (MRM) mode. The precursor and product ions generated were: AA-Su-Saq 929.5/266.2; Su-Saq 771.0/266.2; Saq 671.4/266.2; Verapamil 455.3/155.2. The turbo ion spray setting and collision gas pressure were optimized (IS voltage: 5500 V, temperature: 300 °C, nebulizer gas: 40 psi, curtain gas: 40 psi). Nitrogen was used as collision gas for MS/MS. Other ion source parameters employed were: declustering potential (DP): 95 V; collision energy (CE): 70 V; entrance potential (EP): 8.5 V; and collision cell exit potential (CXP): 4.0 V. Peak areas for all components were automatically integrated by using Analyst™ software, and the peak area ratios (area of analytes to area of IS) were plotted versus concentration by weighted linear regression. Analytical methods were developed and validated for Saq, Saq-Su and AA-Su-Saq. Linearity was obtained over a broad concentration range, Saq: 5 nM to 5 μM ($r^2 > 0.997$); Saq-Su: 10 nM to 5 μM ($r^2 > 0.994$); AA-Su-Saq: 20 nM to 10 μM ($r^2 > 0.992$). Assay accuracy varied from 85.0 to 112.0% and precision ranged from 6.0 to 14.0%. These methods generated rapid and reproducible results.

2.12. Data analysis

The cumulative amount of drug or prodrug is calculated by Eq. (1).

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

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

Transepithelial transport permeability P (P_{A-B} or P_{B-A}) of AA and AA-Su-Saq was calculated according to Eq. (2):

$$P = \left(\frac{d(TR_{cum})}{dt}\right) \times \frac{1}{A} \times \frac{1}{C_0} \quad (2)$$

$d(TR_{cum})/dt$ is transport rate, which was obtained from the slope of the cumulative amount (TR_{cum}) – time profile. A is the surface area of the Transwell® insert, and C_0 is the donor concentration. Permeability values are expressed as centimeters per second (cm/s).

2.13. Statistical analysis

All experiments were conducted at least in triplicate and results were expressed as mean ± SD. Statistical comparisons of mean values were performed with one-way analysis of variance (ANOVA) or Student's *t*-test (GraphPad INSTAT, version 3.1). $^*P < 0.05$ was considered to be significant.

3. Results

3.1. Apparent aqueous solubility

Aqueous solubility of Saq (mesylate salt) and AA-Su-Saq was determined to be 1.75 ± 0.29 and 4.91 ± 0.40 mM. Solubility of AA-Su-Saq was higher than that of Saq (mesylate) by 2.8 fold.

3.2. Stability of AA-Su-Saq in DPBS buffer and Caco-2 cell homogenates

Since DPBS (pH 7.4) is the buffer used for uptake and transport experiments, the stability of the prodrug in this buffer system was first determined. Stability of AA-Su-Saq was determined in DPBS and Caco-2 cell homogenates for 12 h. The slope of the line of "*Ln* (percentage prodrug remaining) versus time" plot was employed to calculate the degradation rate constants. The half lives ($t_{1/2}$) of AA-Su-Saq were obtained as 9.65 ± 0.30 and 5.73 ± 0.74 h in DPBS and Caco-2 cell homogenate, respectively (Table 1).

3.3. Cytotoxicity study

Results obtained from cytotoxicity study are depicted in Fig. 1. Blank medium was used as negative control and doxorubicin (1 mg/mL) was used as positive control. After 6 h incubation, it was found that 10–200 μM prodrug solutions were not cytotoxic but higher concentration i.e. 400 μM of AA-Su-Saq exhibited significant cytotoxicity. Low concentration of AA-Su-Saq (25 μM) and short-term exposure (3 h) in transport studies may not show any cytotoxicity.

3.4. Interaction of Saq and AA-Su-Saq with P-gp in MDCK-MDR1 cells

Uptake of [^{3}H]Saq in MDCK-MDR1 cells was studied in the presence of equimolar concentration (50 μM) of unlabeled Saq and AA-Su-Saq. Cellular accumulation of [^{3}H]Saq increased 2.7 and 1.9

Table 1
Stability of AA-Su-Saq in DPBS buffer and Caco-2 cell homogenates.

	Rate constant (k, h^{-1})	Half life ($t_{1/2}$, h)
DPBS (pH 7.4)	0.0718 ± 0.0022	9.65 ± 0.30
Caco-2 cell homogenates	0.121 ± 0.014	5.73 ± 0.74

Data are shown as mean ± SD ($n = 3$).

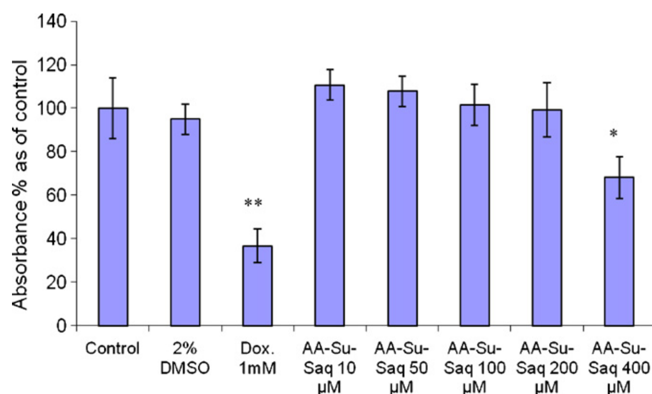


Fig. 1. Cytotoxicity profile of AA-Su-Saq.

fold in the presence of unlabeled Saq and AA-Su-Saq, respectively (Fig. 2). Such enhanced uptake is caused by the inhibition of P-gp mediated efflux of [^{3}H]Saq. However, AA-Su-Saq produced less inhibition to the P-gp mediated cellular efflux compared to unlabeled Saq. These results suggest that AA-Su-Saq exhibits lower binding affinity towards the efflux protein compared to Saq.

3.5. Interaction of AA and AA-Su-Saq with SVCT in MDCK-MDR1 and Caco-2 cells

3.5.1. Uptake of [^{14}C]AA in the presence of AA and AA-Su-Saq

Uptake of [^{14}C]AA (23.5 μM) in the presence of unlabeled AA and AA-Su-Saq (200 μM) was carried out in both MDCK-MDR1 and Caco-2 cells to determine whether the prodrug can be recognized by the vitamin C transporter (SVCT). Uptake of [^{14}C]AA diminished by 65% and 51% in the presence of 200 μM of unlabeled AA and AA-Su-Saq in MDCK-MDR1cells, respectively (Fig. 3). Equivalent concentration of unlabeled AA and AA-Su-Saq produced 66% and 57% inhibition in Caco-2 cells, respectively (Fig. 4). These results indicate that AA-Su-Saq was recognized by SVCT, which is dominantly expressed on the apical side of both MDCK-MDR1 and Caco-2 cells.

3.5.2. Uptake of AA-Su-Saq in the presence of AA

To further examine the hypothesis that AA conjugated prodrug can be recognized by SVCT, uptake of AA-Su-Saq was conducted employing both MDCK-MDR1 and Caco-2 cells. Samples were extracted and analyzed by LC–MS/MS. In the presence of 5 mM AA, cellular accumulation of AA-Su-Saq reduced 27% and 34% in MDCK-MDR1 and Caco-2 cells, respectively (Figs. 5 and 6). These results further indicate that AA-Su-Saq was recognized by SVCT.

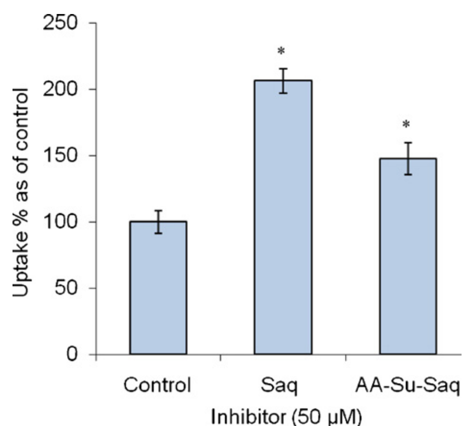


Fig. 2. Effect of Saq and AA-Su-Saq on the uptake of [^{3}H]Saq in MDCK-MDR1 cells.

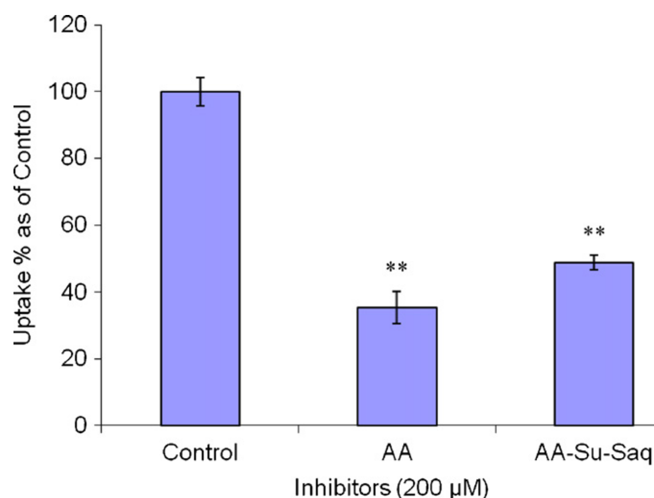


Fig. 3. Inhibition of AA and AA-Su-Saq on the uptake of [^{14}C]AA in MDCK-MDR1 cells.

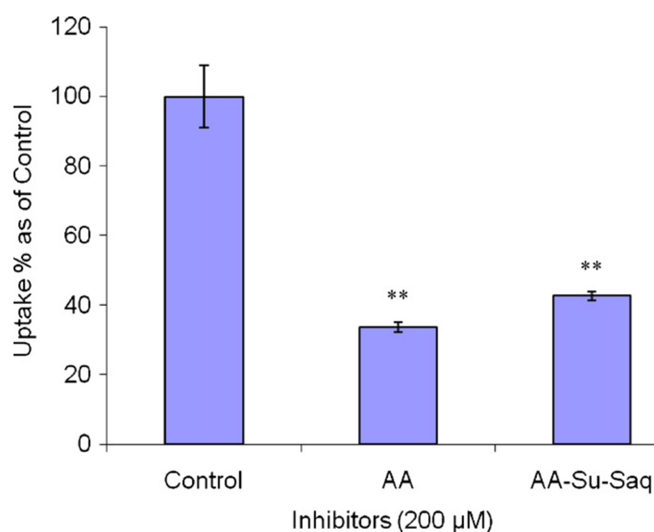


Fig. 4. Inhibition of AA and AA-Su-Saq on the uptake of [^{14}C]AA in Caco-2 cells.

3.6. Transepithelial transport of [^{14}C]AA across Caco-2 cells

In order to compare the permeability of AA in MDCK-MDR1 cells previously reported (Luo et al., 2008), transepithelial trans-

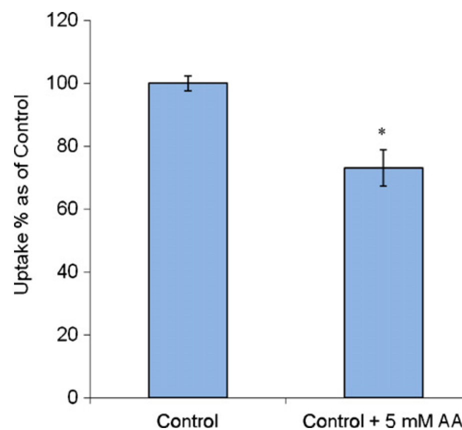


Fig. 5. Uptake of AA-Su-Saq by MDCK-MDR1 cells in the absence and presence of AA.

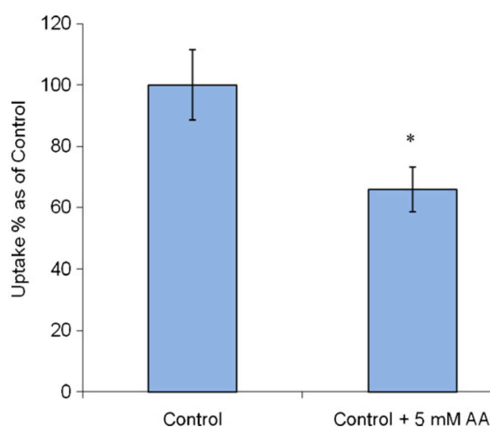


Fig. 6. Uptake of AA-Su-Saq by Caco-2 cells in the absence and presence of AA.

port of [^{14}C]AA was examined across Caco-2 cell monolayers. Apparent permeability (P) in both directions from apical to basolateral (P_{A-B}) and from basolateral to apical (P_{B-A}) was determined from the linear portion of the cumulative amount of drug transported versus time profile (Fig. 7). The permeability values were $(17.6 \pm 0.39) \times 10^{-6}$ cm/s in the A-B direction and $(15.5 \pm 3.11) \times 10^{-6}$ cm/s in the B–A direction, respectively. There was no significant difference between P_{A-B} and P_{B-A}.

3.7. Transepithelial transport of AA-Su-Saq across MDCK-MDR1 and Caco-2 cells

In order to determine the transepithelial permeability of the prodrug, bidirectional transport of AA-Su-Saq was studied across both MDCK-MDR1 and Caco-2 cells. Stability studies of the prodrug in transport buffer and cell homogenates have demonstrated that Saq and Saq-Su can be partially regenerated since degradation of the prodrug may take place during the transport process. Intact prodrug transported and breakdown products formed during the transport process were analyzed. Cumulative amount of drug transported including the prodrug (AA-Su-Saq), regenerated parent drug (Saq) and intermediate (Saq-Su) was plotted as a function of time. Apparent permeability was determined from the linear portion of the cumulative amount of drug transported versus time profile. In comparison, the permeability values of Saq and AA were also summarized in Table 2. Absorptive permeability (P_{A-B}) of AA-Saq-Su was found to be $(2.67 \pm 0.23) \times 10^{-6}$ and $(4.40 \pm 0.57) \times 10^{-6}$ cm/s across MDCK-MDR1 and Caco-2 cells, respectively. The secretive permeability (P_{B-A}) was $(6.53 \pm 0.28) \times 10^{-6}$ and $(7.13 \pm 0.82) \times 10^{-6}$ cm/s across

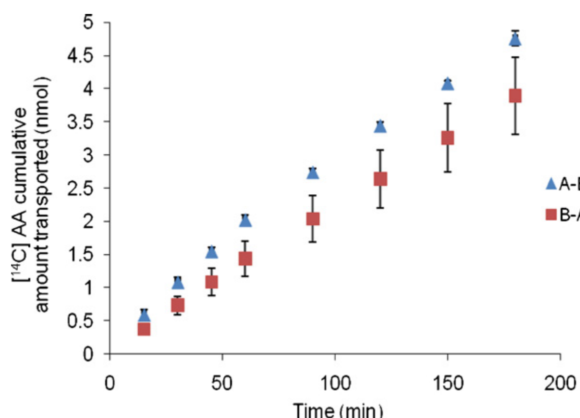


Fig. 7. Transepithelial transport of [^{14}C]AA across Caco-2 cell monolayers.

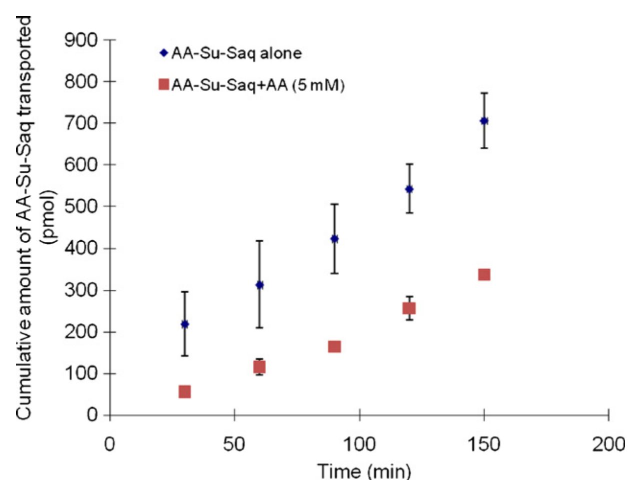


Fig. 8. A–B transport of AA-Su-Saq in the absence and presence of 5 mM AA across MDCK-MDR1 cells.

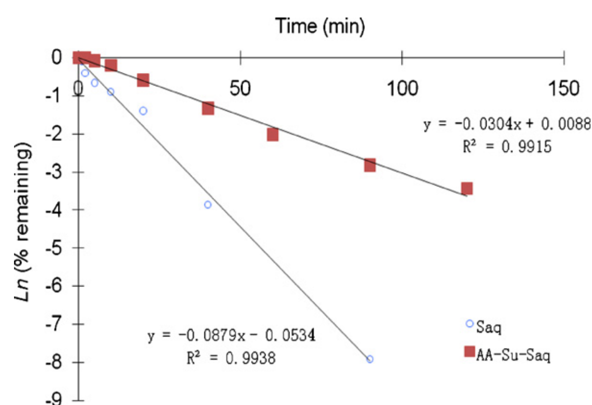


Fig. 9. Metabolic degradation profiles of Saq and AA-Su-Saq in rat liver microsomes.

MDCK-MDR1 and Caco-2 cells, respectively. Efflux index (EI) was 2.44 and 1.62 in MDCK-MDR1 and Caco-2 cells, respectively. These results indicate that prodrug modification significantly enhanced the drug absorptive permeability across the epithelial membranes.

In the presence of 5 mM AA, the absorptive permeability (P_{A-B}) of AA-Su-Saq was found to be $(1.49 \pm 0.06) \times 10^{-6}$ cm/s across MDCK-MDR1 (Fig. 8), which indicates that unlabeled AA caused 44% of inhibition to A–B transport of AA-Su-Saq.

3.8. Metabolism studies

Metabolism of Saq and AA-Su-Saq were studied employing rat liver microsomes. The enzymatic degradation profiles were depicted in Fig. 9. Degradation of Saq and AA-Su-Saq followed apparent first-order kinetics and the rate constants were calculated. Degradation rate constants of Saq and AA-Su-Saq were observed to be 0.0879 and 0.0304 min^{-1}. Half lives of Saq and AA-Su-Saq are 7.88 and 22.3 min. Half life of AA-Su-Saq increased 2.8 fold relative to Saq, which indicates that the prodrug was metabolically more stable.

4. Discussion

We have previously characterized the vitamin C transport system (SVCT) in MDCK-MDR1 cells (Luo et al., 2008). The primary objective of present study was to investigate the viability of targeting this influx transporter to enhance absorption and improve oral bioavailability of Saq by prodrug derivation. The parent drug

Table 2
Permeability values of AA, Saq and AA-Su-Saq across MDCK-MDR1 and Caco-2 cell monolayers.

		$P_{(A-B)}$ (cm/s, × 10^{-6})	$P_{(B-A)}$ (cm/s, × 10^{-6})	Efflux index (EI)
MDCK-MDR1	AA	8.15 ± 0.27	7.97 ± 0.39	0.98
	Saq	0.463 ± 0.025	19.9 ± 1.70	43.0
	AA-Su-Saq	2.67 ± 0.23	6.53 ± 0.28	2.44
Caco-2	AA	17.6 ± 0.39	15.5 ± 3.11	0.88
	Saq	0.93 ± 0.10	20.39 ± 1.53	22.0
	AA-Su-Saq	4.40 ± 0.57	7.13 ± 0.82	1.62

Data are shown as mean ± SD (n = 4).

Saq was attached to AA for several reasons. First, AA is a natural nutrient. Therefore side effects and cytotoxicity can be avoided. It can also serve as an antioxidant (Wilson, 2005). Moreover, AA and analogs may possess antiviral activity (Harakeh et al., 1990; Furuya et al., 2008) and inhibit P-gp expression (El-Masry and Abou-Donia, 2003). In addition, previous studies demonstrated that AA conjugated prodrug of nipecotic acid was recognized by SVCT2 and the prodrug derivation facilitated the drug accumulation and the drug entry to the mouse brain (Manfredini et al., 2002; Dalpiaz et al., 2004). Finally, SVCT transporters responsible for AA uptake are abundantly available in various tissues.

In the present study, the conjugation of Saq with AA was achieved via a natural non-toxic agent succinic acid (Su) as a linker which possesses two carboxylic acid groups. Ideally, AA-Su-Saq can overcome the problems associated with the parent drug Saq, such as low water solubility, low absorptive permeability and bioavailability. Indeed, relative to Saq, AA-Su-Saq showed significantly higher aqueous solubility. Such enhancement in solubility can be attributed to increasing polarity of the conjugated prodrug molecule since AA is highly water soluble. Low oral bioavailability may be caused by many factors including low solubility, susceptible to chemical and enzymatic hydrolysis, and/or excessive cellular efflux and extensive metabolism. Chemical and enzymatic stabilities of AA-Su-Saq were studied in DPBS and Caco-2 cell homogenates. The half life of AA-Su-Saq (5.73 h) in Caco-2 cell homogenates diminished by only about 40% relative to DPBS (9.65 h), confirming that the stability of AA-Su-Saq against hydrolysis by esterases such as carboxylesterases is acceptable. Cytotoxicity studies of the AA-Su-Saq were performed on MDCK-WT cells using MTS assay and the results showed that AA-Su-Saq exhibited no noticeable cytotoxicity within the concentration range studied.

Low oral bioavailability of Saq can be primarily attributed to the cellular efflux mediated by P-gp and extensive metabolism mediated by CYP3A4. MDCK-MDR1 cells are canine renal epithelial cells transfected with human MDR1 genes, which has been widely accepted as an *in vitro* model for screening P-gp substrates. We first studied the uptake of [^{3}H]Saq in MDCK-MDR1 cells in the presence of unlabeled Saq and AA-Su-Saq to examine whether prodrug derivation can lower the interaction of P-gp with Saq. Cellular uptake of [^{3}H]Saq showed 2.7 and 1.9 fold rise in the presence of 50 μM of Saq and AA-Su-Saq, respectively (Fig. 2). Saq and AA-Su-Saq can act as an inhibitor of P-gp and thus uptake of [^{3}H]Saq is accelerated. A differential inhibition by equimolar Saq and AA-Su-Saq suggests that AA-Su-Saq has lower affinity for P-gp than Saq. Prodrug modification of Saq by conjugating with AA via a linker reduced the interaction between P-gp and its substrate. Next, uptake studies were conducted to verify whether AA-Su-Saq is a substrate for SVCT. Previous work indicated that SVCT1 is dominantly expressed on the apical membrane of polarized cells such as MDCK and Caco-2 cells (Maulen et al., 2003; Boyer et al., 2005; Luo et al., 2008). These two cell lines were exploited as *in vitro* models of kidney and intestinal cells to screen the interaction of SVCT with its substrates. Cellular accumulation of [^{14}C]AA decreased about 50–70% in the presence of unlabeled AA and AA-Su-Saq in MDCK-MDR1 cells and Caco-2 cells (Figs. 3 and 4). Such lower uptake of [^{14}C]AA may be attributed to the inhibition of SVCT by unlabeled AA and AA-Su-Saq. These results indicate that AA conjugated prodrug (AA-Su-Saq) was recognized by SVCT. Compared to AA, AA-Su-Saq exhibited slightly lower inhibition to [^{14}C]AA uptake, which indicates that AA-Su-Saq possesses lower affinity for SVCT relative to AA. Recognition of AA-Su-Saq by SVCT was further confirmed by the inhibition of AA on the uptake of AA-Su-Saq in both MDCK-MDR1 and Caco-2 cells (Figs. 5 and 6). Cellular accumulation of AA-Su-Saq significantly diminished in the presence of AA in both cell lines.

Bidirectional transepithelial transport of [^{14}C]AA in MDCK-MDR1 cells was previously reported from our laboratory (Luo et al., 2008). For comparison, we studied the transepithelial transport of [^{14}C]AA in Caco-2 cells (Fig. 7). As shown in Table 2, both absorptive permeability (P_{A-B}) and secretive permeability (P_{B-A}) in Caco-2 cells were about 2 fold higher than that in MDCK-MDR1 cells, which suggests that AA is more permeable in Caco-2 cells. No significant difference was observed between P_{A-B} and P_{B-A} and the efflux index was close to 1 for both cell lines. These results indicate that AA is not a substrate of P-gp.

To further examine whether AA conjugated prodrug can enhance the absorptive permeability of parent drug, transepithelial transport studies across both MDCK-MDR1 and Caco-2 cell monolayers were conducted. P-gp is located on the apical side of the polarized cells. The efflux index (EI), the ratio of secretive permeability P_{B-A} to the absorptive permeability P_{A-B}, typically indicates the involvement of P-gp in the transepithelial transport across the polarized cells. EI values of Saq were reported to be 43 across MDCK-MDR1 cells (Jain et al., 2005) and 22 across Caco-2 cells (Ucpinar and Stavchansky, 2003). Compared to Saq, the EI values of AA-Su-Saq decreased 17.6 and 13.5 fold in MDCK-MDR1 and Caco-2 cells, respectively. In comparison to Saq with the P_{A-B} values of 4.63×10^{-7} cm/s in MDCK-MDR1 (Jain et al., 2005) and 0.93×10^{-6} cm/s in Caco-2 cells (Ucpinar and Stavchansky, 2003), AA-Su-Saq exhibited 5.8 and 4.7 fold higher absorptive permeability in MDCK-MDR1 and Caco-2 cells, respectively (Table 2). Such improvement in the absorptive permeability may be attributed to a combination of increased solubility, lower efflux mediated by P-gp and higher influx mediated by SVCT. When a substrate binds to a nutrient influx transporter, it probably triggers a configuration change. In this process, the substrate may not be freely available in the inner leaflet of the cellular membrane and thus avoids to be recognized by the efflux protein P-gp. Since Saq is known as a good substrate for P-gp, the prodrug modification may reduce the interaction between P-gp and its substrate. The absorptive permeability of AA-Su-Saq significantly decreased in the presence of unlabeled AA (Fig. 8), which further confirmed that the A-B transport of AA-Su-Saq was facilitated by SVCT.

Extensive first-pass metabolism of Saq is another major factor responsible for the low bioavailability besides P-gp mediated efflux. Saq is dominantly metabolized by CYP3A4 in the gut and liver. It is well known that inhibition of CYP3A4 can improve oral bioavailability of Saq. Prodrug modification could modulate

the interaction between CYP3A4 and its substrates which can be exploited to reduce the CYP3A4 mediated metabolism. We conducted the metabolic degradation studies of Saq and AA-Su-Saq using rat liver microsomes. Half life of AA-Su-Saq was significant higher than that of Saq, which suggests that AA-Su-Saq was metabolically more stable than Saq (Fig. 9). These results indicate that the prodrug modification may reduce the affinity between CYP3A4 and its substrate. In conclusion, AA conjugated prodrug modification can be a good strategy to improve the rate and extent of first-pass metabolism of Saq and evade the P-gp mediated efflux and consequently improve the oral absorption of a protease inhibitor such as saquinavir.

5. Conclusion

A novel vitamin C conjugated prodrug, AA-Su-Saq, was successfully synthesized and evaluated in this study. Comparing with parent drug Saq, AA-Su-Saq displayed better solubility. The concentration range of AA-Su-Saq used in present study was found to be devoid of cytotoxicity. Moreover, the prodrug modification by conjugating with AA via a linker increased the absorptive permeability and metabolic stability. Additionally, AA-Su-Saq was shown to be a substrate for vitamin C transporter (SVCT) and capable of bypassing the P-gp mediated efflux and decreasing the rate of CYP3A4 mediated metabolism. In conclusion, our results demonstrate that AA conjugated prodrug modification of Saq targeting SVCT improved its solubility, metabolic stability and absorptive permeability. It suggests that SVCT targeted prodrug approach has the potential to increase the oral absorption and bioavailability of anti-HIV protease inhibitors.

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