Use of the Intestinal Bile Acid Transporter for the Uptake of Cholic Acid Conjugates with HIV-1 Protease Inhibitory Activity

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Purpose. To investigate the ability of the human intestinal bile acid transporter to transport cholic acid conjugates with potential HIV-1 protease inhibitory activity.

Methods. Cholic acid was conjugated at the 24 position of the sterol nucleus with various amino acids and amino acid analogs. The CaCo-2 cell line was used as a model to investigate the interaction of these bile acid conjugates with the human intestinal bile acid transporter. Interaction between the carrier and the conjugates was quantified by inhibition of taurocholic acid transport and confirmed by transport of radiolabelled conjugates in this cell line.

Results. The highest interaction with the transporter, as quantified by inhibition of taurocholic acid transport, occurred when a single negative charge was present around the 24 to 29 region of the sterol nucleus. A second negative charge or a positive charge significantly reduced the interaction. Transport of radiolabelled cholyl-L-Lys-ε-*t*BOC ester and cholyl-D-Asp-β-benzyl ester was inhibited by taurocholic acid. Of all tested compounds, only cholyl-D-Asp-β-benzyl ester showed modest HIV-1 protease inhibitory activity with an IC_{50} of 125 μM.

Conclusions. Cholic acid-amino acid conjugates with appropriate stereochemistry are recognized and transported by the human bile acid transporter and show modest HIV-1 protease inhibitory activity. Transport of these conjugates by the bile acid carrier is influenced by charge and hydrophobicity around the 24 position of the sterol nucleus.

KEY WORDS: AIDS; bile acid carrier; drug conjugates; intestinal transport; HIV-protease inhibition.

INTRODUCTION

During the past few years, carrier-mediated transport mechanisms in the small intestine have been the target of approaches to increase oral bioavailability (1). One of the more interesting transport systems in the gastrointestinal tract is the bile acid carrier. This transport system is an efficient, high capacity system facilitating the daily absorption of 10–20 g bile salts at a more than 95% efficiency rate. Next to transporting its natural substrates, conjugated and unconjugated bile acids, it allows modifications at the 3 and 24 positions (Fig. 1.) of the sterol nucleus (2,3). Modifications at the 3 position, as introduced by Ho (2), are most widely investigated and success has been reported in transporting small peptides conjugated to this position (4,5).

It has been shown that bile acid derivatives have an intrinsic anti-HIV activity *in vitro* (6,7), although the mechanism of action remains unclear. However, a number of compounds with structures similar to bile acids were shown to have partial HIV-1 protease inhibitory activity (P. Ortiz De Montellano, personal communication). Preliminary assessment using a docking program (8) suggests that cholic acid will associate with the HIV-1 protease oriented with the 24 position toward the interior of the protease.

It was the goal of this study to determine the potential for small peptide conjugates at the 24 position of cholic acid to be transported by the ileal bile acid transporter and to determine whether such compounds may be potential HIV-1 protease inhibitors.

METHODS

Materials

The CaCo-2 cell line, obtained from the UCSF cell culture facility, was used between passages 23 and 40. High glucose DMEM, heat-inactivated FBS, NEAA, penicillin-streptomycin, trypsin, HBSS, Hepes buffer and glucose were all obtained from the UCSF cell culture facility. Rat tail collagen (Type I) was obtained from Collaborative Research (Lexington, MA). Taurocholic acid, cholic acid, tetraethylammonium chloride and choline chloride were obtained from Sigma (St. Louis, MO). [^{3}H]-taurocholic acid, [^{3}H]-cholic acid and 1,2-[^{14}C]-polyethylene glycol 4000 (PEG-4000) were purchased from NEN Research Products (Boston, MA). Transwell™ clusters (24.5 mm diameter, 3.0 μm pore size) were from Costar Corporation (Bedford, MA). All other chemicals used were of reagent grade.

Synthesis of Cholic Acid Conjugates

A modification of the synthetic procedure described by Bergström and Norman (9) was used: cholic acid was reacted with isobutyl chloroformate in dioxane in the presence of a tertiary amine to form a mixed anhydride. The amino acid analog to be conjugated to cholic acid was solubilized by titration with sodium carbonate or triethyl amine and reacted with the mixed anhydride in an ice bath for 2 hours. The generated conjugates were precipitated with hydrochloric acid and recrystallized in ethyl acetate. The conjugates were further purified using a Dynamax 300A preparative C-18 column using a gradient of acetonitrile and water with 0.1% trifluoro acetic acid. Cholyl-ε-*t*BOC-L-Lys was not stable under these conditions and was purified by chromatography on silica gel (mesh 60) with a mixture of chloroform, methanol and ammoniumhydroxide (65:35:5) as the mobile phase. The identity of the compounds was determined by mass spectrometry and NMR. The structural formulas of the synthesized compounds together with their calculated and experimental masses are given in Figure 1.

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ABBREVIATIONS: AIDS, adult immunodeficiency syndrome; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; Hepes, (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonate]); HIV, human immunodeficiency virus; NEAA, non-essential amino acid; PEG, polyethylene glycol; *t*BOC, *tert*-butoxycarbonyl.

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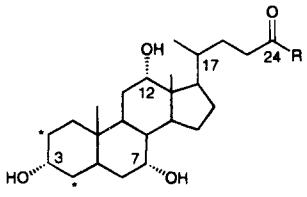

	Name	-R	M_W Calc.	Exp.
A	Cholic Acid	-OH		
A	Taurocholic Acid	-NH-CH$_2$-CH$_2$-SO$_3$H		
	Cholyl-D-Alanine	-NH-CH(COOH)-CH$_3$	479.3	480.3 [M+H]
	Cholyl-L-Serine	-NH-CH(COOH)-CH$_2$-OH	495.3	494.3 [M-H]
	Cholyl-L-Cysteine	-NH-CH(COOH)-CH$_2$-SH	511.3	510.3 [M-H]
B	Cholyl-D-Aspartic Acid	-NH-CH(COOH)-CH$_2$-COOH	523.3	524.2 [M+H]
C	Cholyl-ε-D-Lysine	-NH-CH(COOH)-(CH$_2$)$_4$-NH$_2$	536.4	537.3 [M+H]
C	Cholyl-L-Lysine	-NH-CH(COOH)-(CH$_2$)$_4$-NH$_2$	536.4	535.4 [M-H]
	Cholyl-D-Ala-D-Ala	-NH-CH(CH$_3$)-CO-NH-CH(COOH)-CH$_3$	550.7	551.6 [M+H]
	Cholyl-D-Asp-α-benzyl ester	-NH-CH(COOH)-CH$_2$-CO-O-CH$_2$-C$_6$H$_5$	613.4	614.3 [M+H]
	Cholyl-D-Asp-β-benzyl ester	-NH-CH(COOH)-CH$_2$-CO-O-CH$_2$-C$_6$H$_5$	613.3	614.3 [M+H]
	Cholyl-L-Glu-γ-benzyl ester	-NH-CH(COOH)-CH$_2$-CH$_2$-CO-O-CH$_2$-C$_6$H$_5$	627.4	626.3 [M-H]
	Cholyl-L-Lys-ε-tBOC ester	-NH-CH(COOH)-(CH$_2$)$_4$-NH-CO-O-C(CH$_3$)$_3$	636.3	637.4 [M+H]

Fig. 1. Structural formulas of the compounds synthesized for this study. Important carbon atoms in the sterol nucleus are indicated by IUPAC numbering. An asterisk indicates the position of the tritium label in radiolabelled compounds. The calculated and experimental molecular mass of the conjugates is indicated next to their structural formulas.

Cell Culture

Caco-2 cells were grown in 175 cm^2 culture flasks (Costar Corporation) in culture medium consisting of high-glucose (4.5 mg/l) DMEM supplemented with FBS (10%), NEAAs (1%), penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were maintained at 37°C in an atmosphere of 5% CO_2. At approximately 80% confluence, cells were trypsinized and plated at a density of 64,000 cells/cm^2 on Costar Transwell™ polycarbonate membranes, previously coated with rat tail collagen. The cell culture medium, 1.5 ml apical side and 2.5 ml basolateral side, was replaced every other day for the first week and daily thereafter. Experiments were performed on 23 to 31 days after seeding.

Transepithelial Transport Studies

The transepithelial transport of cholic acid, taurocholic acid, cholyl-L-Lys-ε-*t*BOC ester and cholyl-D-Asp-β-benzyl ester was determined in CaCo-2 cells at 37°C. Cell monolayers were washed twice with modified HBSS (containing 25 mM glucose and 10 mM Hepes buffer; pH 7.4) and allowed to equilibrate for 20 min. at 37°C. Studies were initiated by replacing the solution on the apical side with 1.5 ml test solution in HBSS (4, 10, 26, 64, 160 and 400 μM cholic acid containing 2.5 μCi [^{3}H]-cholic acid; 4, 12, 35, 105, 211 and 421 μM taurocholic acid containing 2.5 μCi [^{3}H]-taurocholic acid; 20 μM [^{3}H]-Cholyl-L-Lys-ε-*t*BOC ester; or 30 μM [^{3}H]-Cholyl-D-Asp-β-benzyl ester). Samples of 500 μl were taken from the basolateral side at designated times and replaced with fresh 500 μl transport solution. The amount of radiolabelled material in the samples was determined using a Beckman LS-5801 liquid scintillation counter. The transport rate was calculated by linear regression from the amount recovered on the basolateral side during the experiment. The apparent transport rate (J_{app}) was subsequently fitted to a transport equation with a linear term:

$$J_{app} = \frac{J_{max} \cdot C}{K_T + C} + P_m \cdot C$$

where J_{max} is the maximum rate of transport, K_T the concentration where the transport is 50% of maximum, C the average bile acid concentration during the collection period and P_m is the passive permeability constant (l/min·mg protein). The data was fitted using the Minim program (version 3.0.8; R. P. Purves, Dunedin-NZ) using a least sum of squared errors and Hartley's interpolation.

Inhibition Studies

Cell monolayers were rinsed and preincubated as described above for transport studies. Studies were initiated by replacing the solution on the apical side with 1.5 ml 3μM [^{3}H]-taurocholic acid in presence or absence (control) of a 100-fold excess of a bile acid conjugate. The concentration of the bile acid analogs [^{3}H]-Cholyl-L-Lys-ε-*t*BOC ester and [^{3}H]-Cholyl-D-Asp-β-benzyl ester was 20 and 30 μM, respectively. Percent inhibition of transepithelial transport was calculated by comparing the apparent transport rate in the presence of an inhibitor against the control.

Influence of Culture Time on Bile Acid Carrier Expression

To study the time of maximum bile acid transporter expression, transport studies were carried out with 3 μM [^{3}H]-taurocholic acid between 16 and 30 days after seeding on Transwell™.

Sodium Dependency and Monolayer Integrity

To study the sodium dependency of transport, HBSS was modified by replacing NaCl with an equal molar concentration of choline chloride or tetraethyl-ammonium (TEA) chloride. All other sodium salts were replaced with the corresponding potassium salts in these modified solutions. To minimize the alteration in potassium concentration, KCl was deleted from the buffer. Glucose (25 mM) and Hepes buffer (10 mM) were

added to the modified solutions as in the regular transport solution. Prior to starting transport studies the inserts were carefully washed with the sodium-free buffer.

The integrity of the cell monolayers was determined by checking the paracellular transport of 1 μCi[^{14}C]-PEG-400. If transfer of PEG-4000 reached values of 0.5%/hour, the results were discarded due to suspected membrane leakage.

HIV-1 Protease Inhibitory Activity

The activity of conjugates as potential protease inhibitors was measured as the *in vitro* ability to inhibit HIV-1 and HIV-2 proteases cleavage of the decapeptide ATLNFPISPW, corresponding to the HIV-1 C-terminal autoprocessing site. The two pentapeptides generated were measured by HPLC (8).

Protein Concentration

The cells were lysed by placing the cell cultures filters in a 0.1% triton-X solution. An aliquot was used for measurement of total protein concentration using the BCA protein assay (Pierce Rockford, IL). Background values were determined from unseeded filters.

RESULTS

Influence of Time on Bile Acid Carrier Expression

The CaCo-2 cells appeared to have grown to confluence by day 16, as the PEG leakage did not vary more than 0.17 ± 0.04 %/hour beyond this day. The bile acid transporter was well expressed by day 16 with a transport rate of taurocholic acid of 14.0 ± 2.1 %/hour. The ability to transport taurocholic acid increased only slightly up to day 29 (data not shown) indicating that differentiation of the cells was essentially completed by day 16. Transport was linear with time after an initial lag-time of approximately 15 min. The transport of taurocholic acid was highest at low passage number (<30) and appears to decline at higher passage numbers. Only CaCo-2 cells with passage number between 23 and 40 were, therefore, used during the studies.

Sodium Dependency of Bile Acid Transport

Transepithelial transport of [^{3}H]-taurocholic acid by CaCo-2 cells was strongly dependent on the presence of Na^+ in the solution. Replacement of Na^+ with choline or TEA decreased the transport of taurocholic acid by 97.2 ± 0.40% and 97.2 ± 1.1%, respectively (Fig. 2). These results are in good agreement with the reduction of 95% reported by Chandler and colleagues (10) who used passage number 48–59 but substantially larger than the values of 27% reported by Hidalgo and Borchardt (11) who used passage number 59–70.

Transepithelial Transport of Cholic and Taurocholic Acid

The transport of taurocholic acid as a function of concentration followed a traditional carrier-mediated transport relationship with a J_{max} of 151 ± 13 pmol/min/mg of cellular protein and a K_T of 74 ± 21 μM (data not shown). No linear transport term was found for taurocholic acid. The K_T is similar to the values of 65 and 50 μM reported by Chandler and co-workers (10) and Hidalgo and Borchardt (11), respectively. The J_{max} found in these studies are ten times higher than results reported by Hidalgo and Borchardt (11) and one fifth of the value reported by Chandler and colleagues (10). The total protein concentration of 832 μg/filter is similar to the value reported by Chandler and colleagues (10). The transport of cholic acid showed a similar saturable transport as taurocholic acid with

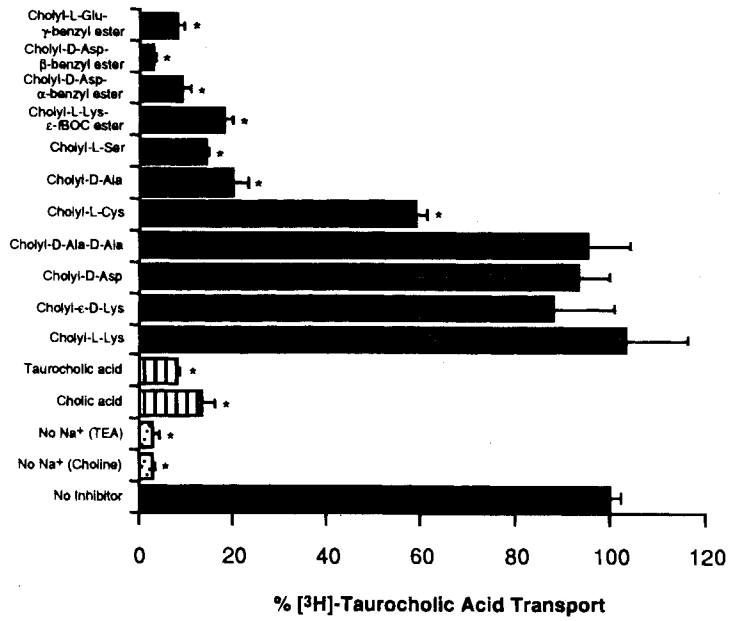

Fig. 2. Relative transport of 3 μM taurocholic acid (control, black bar) in CaCo-2 cells in the presence of natural substrates (striped bars), absence of sodium (dotted bars), and the presence of various cholic acid conjugates (gray bars); n = 4–6.

a J_{max} of 70 ± 19 pmol/min/mg cellular protein and a K_T value of 29 ± 16 μM (data not shown). In contrast to taurocholic acid, cholic acid also had a non-saturable term (P_m in the relationship above) with a value of 0.52 ± 0.05 nl/min·mg cellular protein. Similar linear terms for cholic acid have also been reported by Aldini and co-workers (12). To minimize the influence of passive diffusion on apparent transepithelial transport, taurocholic acid was used in all inhibition studies.

Inhibition of Taurocholic Acid Transport by Bile Acid Conjugates

The ability of bile acid conjugates (Fig. 1) to inhibit taurocholic acid transport in CaCo-2 cells varied from being undetectable for cholyl-L-lysine to an almost complete inhibition by cholyl-D-Asp-β-benzyl ester (Fig. 2). Strong inhibition of taurocholic acid transport was observed with taurocholic acid itself, cholic acid and the cholic acid conjugates of ε-*t*BOC-L-lysine, D-alanine, β-benzyl-D-Asp and α-benzyl-D-Asp. All of these compounds have a single negative charge between position 24 and 28 (position 25 and higher being part of the conjugated side chain). The D-aspartic acid conjugate, which bears two negative charges in this region, showed only marginal inhibitory activity. Addition of a positive charge decreased the inhibition dramatically (Fig. 2: cholyl-ε-D-lysine and cholyl-L-lysine). Addition of an aromatic (hydrophobic) group appears to increase the affinity for the transporter (Fig. 2: cholyl-D-Asp: α- and β-benzyl esters; Cholyl-L-Glu-γ-benzyl ester). An extra hydroxyl group around the 24 position does not influence the affinity for the bile acid transporter (Fig. 2: cholyl-L-serine), whereas a sulfhydryl group seems to reduce affinity (Fig. 2: cholyl-L-cysteine). The maximum length of the side-chain cannot be estimated from these studies, but can at least be 14 Å long.

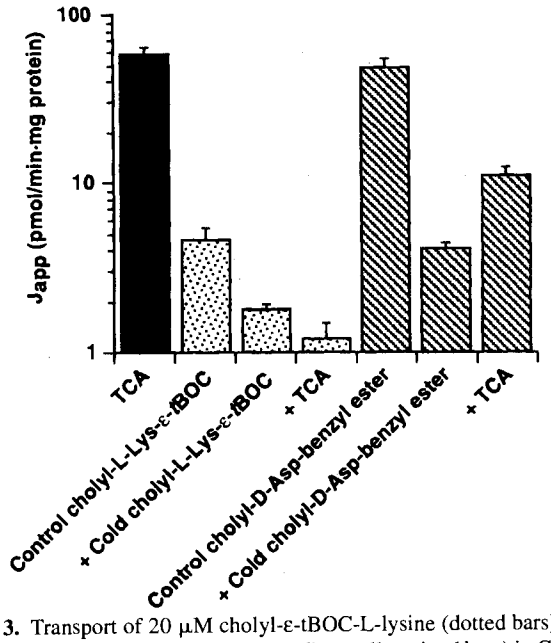

Fig. 3. Transport of 20 μM cholyl-ε-tBOC-L-lysine (dotted bars) and 20 μM cholyl-D-Asp-β-benzyl ester (diagonally striped bars) in CaCo-2 cells (n = 4–8). The transport of 20 μM taurocholic acid (TCA, black bar) from the same batch of cells is also given. The transport of both conjugates was significantly suppressed in the presence of a 100-fold excess of taurocholic acid (+TCA) or non-radioactive (cold) compound. Note the logarithmic scale of the ordinate.

Transepithelial Transport of Radiolabelled Bile Acid Conjugates

Both of the two radiolabelled cholic acid conjugates were readily transported and suppressed by excessive concentration of non-radiolabelled compound or taurocholic acid (Fig. 2). [^{3}H]-cholyl-L-Lys-ε-*t*BOC ester (20 μM) was transported at a rate of 4.63 ± 0.84 pmol/min·mg cellular protein. [^{3}H]-Taurocholic acid at 20 μM using cells from the same seeding had a transport rate of 30.9 ± 4 pmol/min·mg cellular protein. The transport of [^{3}H]-cholyl-L-Lys-ε-*t*BOC ester was suppressed by 74% (to 1.80 ± 0.12 pmol/min·mg protein) with 420 μM non-radiolabelled cholyl-L-Lys-ε-*t*BOC ester and by 83% (to 1.20 ± 0.30 pmol/min·mg protein) with 420 μM taurocholic acid. [^{3}H]-cholyl-D-Asp-β-benzyl ester (30 μM) was transported at a rate of 48.5 ± 5.7 pmol/min·mg cellular protein. Taurocholic acid at 30 μM using cells from the same seeding had a transport rate of 58.6 ± 7.0 pmol/min·mg protein. The transport of [^{3}H]-cholyl-D-asp-β-benzyl ester was suppressed by 92% (to 4.1 ± 0.3 pmol/min/mg cellular protein) with 390 μM non-radiolabelled cholyl-D-Asp-β-benzyl ester and by 77% (to 11.0 ± 1.5 pmol/min·mg cellular protein) with 370 μM taurocholic acid. Less than 3% of the radioactivity on the basolateral side at the conclusion of the experiments were found to be associated with cholic acid or other putative breakdown products.

HIV-1 Protease Inhibition of Bile Acid Conjugates

Six of the conjugates were tested for HIV-1 protease inhibitory activity (cholyl-D-Asp-α-benzyl ester, cholyl-D-Asp-β-benzyl ester, cholyl-D-lysine, cholyl-L-lysine, cholyl-L-Alanine and Cholyl-D-Ala-D-Ala). In the concentration range tested, 10–125 μM, only β-benzyl-D-asp-cholic acid showed a significant activity (Fig. 4) with an IC_{50} of 125 μM.

DISCUSSION

In this study, we investigated the possibility of creating hybrid molecules that have both high oral availability and an intrinsic pharmacological activity, i.e. HIV-1 protease inhibi-

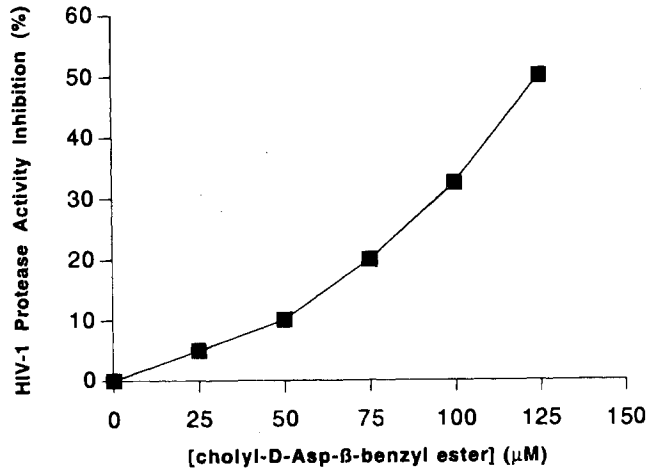

Fig. 4. Relative inhibition of HIV-1 protease by cholyl-D-Asp-β-benzyl-ester. The activity of the HIV protease activity in the absence of β-benzyl-D-asp cholic acid was set at 100%.

tion. Oral absorption can be manipulated in many ways, but the use of carrier-mediated transport mechanisms in the intestine has recently drawn much attention. We chose to target to the intestinal bile acid carrier, a transporter with a high capacity and low structural selectivity. Previously, the 3-position of the sterol nucleus has been successfully utilized to create prodrugs of renin-inhibitory peptides (13), chlorambucil (4), and HMG-CoA reductase inhibitors (14). The concept of creating hybrid molecules by replacing the C-17 side chain has been applied by Kramer and co-workers (14) to target HMG-CoA reductase inhibitors to the hepatic bile acid transporter. In this study, we coupled amino acids and amino acid analogs to the 24-position of the sterol nucleus in order to create hybrids that would be recognized by the intestinal bile acid carrier *and* exert HIV-1 protease inhibitory activity.

To investigate the interaction of cholic acid hybrids with the human intestinal bile acid carrier we characterized bile acid transport in the CaCo-2 cell line. These cells, which undergo spontaneous enterocytic differentiation when grown to confluence in cell culture, were previously shown to express the human bile acid transporter (10,11). Our functional characterization showed that bile acid transport in CaCo-2 cells is a sodium-dependent, active process following carrier-mediated transport kinetics and can be inhibited by structural analogs, which is in good agreement with the established literature.

The results confirm studies by other investigators that the bile acid transporter binds a number of modified bile acids and is capable of transporting, at least some, of these compounds (4,5,13). A number of restrictions exist with regard to the ability of the bile acid transporter to interact and transport bile acid conjugates. We have confirmed and extended observations in the literature (3,15) that ionization and the location of the ionized group(s) are important for interaction with the transporter. It is also likely that the size of the conjugated side chain at the 24 position must be within certain size for the bile acid conjugate to be transported. However, at this point it is not clear what this limit is—only that transport can occur with a side chain of at least of 14 Å length. Hydroxyl groups around the 24 position, such as in cholyl-L-serine, do not seem to interfere with affinity for the bile acid carrier, whereas a sulfhydryl group does (cholyl-L-cysteine), probably due to the partly acidic nature of the sulfhydryl group in cysteine. Large hydrophobic moieties, such as the benzyl group in esters of cholyl-aspartic and -glutamic acid, appear to increase binding to the transporter.

We anticipated from computer-assisted docking analysis that the bile acid would fit into the binding pocket of the HIV-1 protease (16). The activity of cholyl-D-Asp-β-benzyl ester is similar to the activity of other lead compounds (16) but lower than many of the compounds currently being investigated. Our preliminary computer evaluations using DOCK (8) suggest that an elongation of the side chain has the potential for increasing the HIV protease inhibitory activity of these conjugates and might be a fruitful area for future development. Theoretically, this may alleviate the need for separating the bile acid from the active side chain prior to activity in the body. Moreover, the bile acid moiety has surfactant characteristics and may actually enhance cellular penetration after uptake (16). A potential drawback of this approach is that the conjugates may concentrate in the liver due to hepatic bile acid transporters. If the compound concentrates in the liver a separation of the active side chain from the bile acid may be necessary to produce a practical product, even though the HIV-1 activity may be maintained or enhanced by the presence of the bile acid. In addition, issues relating to stability in the G.I. tract and to bioavailability would also have to be assessed and resolved.

In conclusion, the use of the bile acid-amino acid hybrids as compounds with high intestinal absorption and HIV-1 protease inhibitory activity seems feasible. The side chain can be at least 14 Å long and still be transported. The use of cholic acid as both a means of transporting small peptides with HIV activity to increase gastrointestinal absorption as well as the use of intact conjugates as inhibitors appears as an attractive potential for development of hybrid compounds with both high oral absorption and HIV-1 protease inhibitory activity.

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