Multifunctional Bile Acid Derivatives as Efficient RNA Transporters (Carriers)

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Abstract
RNA interference (RNAi) is an emerging technology that is revolutionizing many strategic approaches to biochemical pathway analysis, drug discovery, and therapy. The small interfering RNA (siRNA) can be used to suppress a specific target. Even though these molecules may have potential and strong utility function, many limitations make their clinical application difficult, including delivery problems, side effects due to off-target actions, disturbance of physiological functions of the cellular machinery involved in gene silencing, and induction of the innate immune response. This paper focuses on efficient RNA transport using designed guanidinium steroid carriers. It presents the synthesis of guanidine substituted bile acids (cholic acid, deoxycholic acid and lithocholic acid) by a simple method with moderate yield. The guanidine substituted compounds are shown to be very efficient tools in generating cellular delivery vehicles as determined by in vitro studies using parallel artificial membrane permeability (PAMPA) assay.

Keywords
Bile acids; Guanidine; RNA transport; Parallel artificial membrane permeability; siRNA

Introduction
RNAi is a sequence-specific endogenous gene-silencing mechanism conserved in many organisms to regulate gene expression [1-10]. Two types of small ribonucleic acid (RNA) molecules – microRNA (miRNA) and small interfering RNA (siRNA) – are central to RNA interference. RNAs are the direct products of genes, and these small RNAs can bind to other specific messenger RNA (mRNA) molecules and either increase or decrease their activity, for example by preventing an mRNA from producing a target protein. The specificity of RNAi also allows the inhibition of previously undruggable targets. The ubiquity of the RNAi pathway within the body and the ease with which siRNA can be used to suppress a specific target of interest make siRNAs a promising class of molecules for the treatment of cancer, viral infections, ocular disorders, and genetic diseases [5,11,12]. However, one serious limitation for clinical application is a delivery problem. For an active distribution of a drug, it is necessary to develop its carrier loading, targeting, and transport using toolproof drug delivery. Despite common properties delivery of siRNA faces specific challenges due to apparent differences in size, stability of the formed nucleic acid complexes, the location and mechanism of action [13-28]. The ability of a drug or probe to cross a biological barrier has historically been viewed to be a function of its intrinsic physical properties. This view has largely restricted drug design and selection of agents within a narrow logP range. Molecular transporters offer a strategy to circumvent these restrictions. For example, guanidinium-rich transporters (GRTs), typically highly water-soluble conjugates are able to readily pass through the non-polar membrane of a cell and even cross tissue barriers. For many applications, the transporter-drug conjugate can be designed as a prodrug, with a linker that is chemically or biochemically cleaved after passage through a barrier, allowing for release of the free drug in targeted cells or tissue. Guanidinium groups are well-suited for adherence to cell surfaces bearing negatively-charged groups. The association would allow for increased residence time and thereby favor internalization. The association, though relatively strong, must be reversible to allow release after entry. Though there are many possible mechanisms for GRT uptake, implication of endocytosis is pivotal. To be therapeutically active, GRT conjugate has to escape from endosomes. Such attempt has been made including the cargo release from transporter. Once endosomal escape has been achieved in many cases it is still necessary to release the cargo from the transporter [13]. The tissue uptake of GRTs is of critical importance; therefore the potential of polyguanidinium transporters to enhance bioavailability of biotinylated oligoarginine in mice has been investigated [14]. GRTs have been shown to penetrate many types of tissue, calling attention to their selectivity. Such can be achieved by local administration or by targeted delivery system. One strategy to achieve selectivity is the use of local administration. This has potentially wide application as lungs, buccal, ocular, skin and many other sites are readily reached by direct administration of a drug. GRTs show uptake in lung tissue when inhaled, as well as penetration of both the epidermal and dermal layers when applied topically. In addition to local delivery, a targeted delivery system can also be utilized to achieve specificity. This targeted delivery approach has been demonstrated by using a prodrug strategy in which a transporter is transiently disabled by intramolecular interaction with a polycarboxylate connected to the transporter through a peptide link. The guanidinium-rich molecular transporters facilitate or enhance the delivery of a variety of cargos, including small molecules, metals, imaging agents, peptides, plasmids, and proteins across biological barrier [15,16]. Therefore, we investigated whether guanidinium-rich side chains can also bind siRNA through electrostatic and hydrogen-bonding associations and hydrophobic side chains to facilitate packing and cellular entry as effective siRNA complexation and delivery vehicles. Similarly, steroids represent an important class of natural products due to their high ability to penetrate cells and bind to nuclear and membrane receptors. Bile acids are steroidal acids found predominantly in the bile of mammals and other vertebrates. The cleavage of the peptide bond is presumably carried out in nature by an enzyme. In the laboratory, it is accomplished by an alkaline hydrolysis. The fact that minor changes in steroid structures can cause extensive changes in biological activity intrigued medicinal chemists. Naturally occurring steroid nuclei have been modified in several ways with the aim to find more active compounds, free from undesirable or without harmful side effects,
and of recognising and recognize structural and stereochemical features required for the display of specific and selective physiological activity. The replacement of one or more functional groups in bile acid molecule brings notable modifications of its biological activity. Local delivery of siRNA is advantageous for tissues that are external and/or locally restricted including ocular, epidermal, pulmonary, colonic, and pancreatic tissue. Additionally, local delivery may be suitable for non-invasive therapies that require patient administration, such as eye drops and nasal sprays [17]. Focus of this work is on combination of guanidine moiety (specific for phosphodiester binding) with hydrophobic steroid skeleton. Such structural fragments combined in carrier design should provide, not only stabilization of RNA, but also facilitate transmembrane transport.

**Result and Discussion**

**Chemistry**

We prepared guanidine substituted bile acids at C-3 position of methyl lithocholate, methyl deoxycholate and methyl cholate by using thioureia as reagent. In the preparation of guanidine substituted bile acids we follow several steps and used lithocholic acid, deoxycholic acid and cholic acid as starting material. We stabilize the reaction by using lithocholic acid. We esterified lithocholic acid and then C-3 hydroxy group is converted into leaving group by using methane sulfonyl chloride, which was confirmed by 1H NMR and 13CNMR as a corresponding peak at 2.98 for OMS. The OMS substituted lithocholate reacted with sodium azide and then reduced to amine by using triphenylphosphine.

C-3 amino methyl lithocholate reacted with Boc protected thiourea in the presence of HgCl2 from 0°C to RT for 3 h. Then the product is purified by using column chromatography and its structure was confirmed by using 1H NMR and 13CNMR and mass spectroscopy. In this Boc protons appear at 1.5 ppm and NH protons at 8.9 and 11.48 ppm. In 13CNMR C=N of Boc protected guanidine appear at 157.0 ppm. The guanidine was prepared by using TFA for deprotection of Boc. Guanidine compounds are confirmed by 1H NMR and 13CNMR and mass spectroscopy, in 13CNMR guanidine C=N appears at 157.7 ppm and the same procedure followed to methyl deoxycholate and methyl cholate, they are also confirmed by spectroscopic analysis. We also synthesized the C-26 guanidine substituted of lithocholyl ethyl amide, deoxycholyl ethyl amide and cholyl ethyl amide from bile acid esters of lithocholic acid, deoxycholic acid and cholic acid. Methyl lithocholate react with ethylene diamine and formed amido amine, which was reacted with Boc protected thiourea in presence of HgCl2 from 0°C to RT for 4 h. Then the product is purified by using column chromatography and its structure was confirmed by using 1H NMR and 13CNMR. In this Boc protons appear at 1.5 ppm and NH protons at 8.9,25 and 11.48 ppm. In 13CNMR C=N of guanidine, Boc C=O and NHCO appear at 152.75, 156.96, and 174.12 ppm. The guanidine was prepared by using TFA for deprotection of Boc. The structures of guanidine compounds are confirmed by 1H NMR and 13CNMR and mass spectrometry, in 13CNMR guanidine C=N appears at 157 ppm and the same procedure followed to deoxycholyl ethyl amide and cholyl ethyl amide, they are also confirmed by spectroscopic analysis.

**Bioactivity**

In our experiment we use guanidine substituted bile acids in 5% methanol solution. Further, we use donor phase 300 µL solution, in this 275 µL PBS and 25 µL RNA. In acceptor, we use 200 µL PBS for control experiment. We incubated the plates for 6 h, and then we determined acceptor volume by UV-Vis spectroscopy for control experiment. In guanidine experiment, change the volume of PBS as 250 µL, 25 µL as guanidine bile acids and 25 µL RNA then incubated the plates for 6 h, then we calculated acceptor volume by UV-Vis spectroscopy, binding constant (K) were calculated from changes in absorbance of steroids (A) by nonlinear regression using the program letagroup spefo 2005 the values are given in the table below. We find good results for guanidine at C-26 of deoxycholyl ethyl amide and cholyl ethyl amide. Permeation of our membrane layer is strongly dependent on the pH, which used in the flux measurements. This indicates the importance of different pH values in permeation assays used in human bioavailability prediction [25].

**Conclusion**

We synthesise guanidine substituted cholic acid, chenodeoxycholic acid, deoxycholic acid, and lithocholic acid. We studied RNA transport by using PAMPA assay with guanidine substituted bile acids, RNA from torula yeast and phosphate buffered saline (PBS). Our guanidine substituted compounds are proven to be a very efficient strategy to generate cellular delivery vehicles by studying PAMPA assay as in-vitro studies.

**Experimental Section**

Chemicals were from Sigma Aldrich and used without further purification. All solvents used for the spectroscopic and other physical studies were reagent grade and were further purified by literature methods (24). 1H and 13C NMR spectra were recorded in CDCl3 and MeOD-solutions on a Bruker Avance DRX 500 MHz (also 300 MHz 1H NMR data are given?) spectrometer equipped with a 5 mm diameter, broad band inverse detection probehead operating at 500.13 MHz in 1H and 125.77 MHz in 13C. The 1H and 13C NMR chemical shifts were expressed in parts per million (ppm) with reference to tetramethylsilane (TMS) as an internal standard. LC mass spectra were recorded on a Jeol SX 102 DA/600 mass spectrometer. Elemental analysis was performed on Thermo Finnigan Instrument.

**Synthesis**

Synthesis of methyl 3α-hydroxy-5β-cholane-24-oate (4), methyl 3α,12α-dihydroxy-5β-cholane-24-oate (5) and methyl 3α, 7α, 12α-trihydroxy-5β-cholane-24-oate (6): Compounds 4-6 [17], 7-9 [29] 16-21 [19,22], 22-24 [20], and N,N′-Bis-tert-butoxycarbonylthiourea [23] were synthesized using previously reported methods. [17].

Methyl 3α-hydroxy-5β-cholane-24-oate (4): White solid; yield=98%; 1H NMR (CDCl3, 300 MHz): δ 0.64 (s, 3H, CH3-18), 0.92 (s, 3H, CH3-19), 0.98 (d, J=6.0 Hz, 3H, CH3-21), 3.66 (s, 3H), 3.98 (br s, 1H, CH-3).

Methyl 3α,12α-dihydroxy-5β-cholane-24-oate (5): White solid; yield=95%; 1H NMR (CDCl3, 300 MHz): δ 0.68 (s, 3H, CH3-18), 0.91 (s, 3H, CH3-19), 0.98 (d, J=6.0 Hz, 3H, CH3-21), 3.62 (m, 1H, CH2-12), 3.67 (s, 3H), 3.98 (br s, 1H, CH-3).

Methyl 3α,7α,12α-trihydroxy-5β-cholane-24-oate (6): White solid; yield=97%; 1H NMR (CDCl3, 300 MHz): δ 0.68 (s, 3H, CH3-18), 0.89 (s, 3H, CH3-19), 0.98 (d, J=6.0 Hz, 3H, CH2-21), 3.57 (m, 1H, CH-7), 3.67 (s, 3H), 3.89 (br s, 1H, CH-12), 4.01 (br s, 1H, CH-3).

Synthesis of methyl-3α -mesoseryl-5β-cholane-24-oate (16), methyl-3α -mesoseryl-5β-cholane-24-oate (17),

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Methyl-3α-mesyloxy-7α, 12α-dihydroxy-5β-cholane-24-oate (16): White solid; yield=82%; 1H NMR (CDCl3, 300 MHz): 0.69 (s, 3H, CH3-18), 0.97 (s, 3H, CH3-19), 0.98 (d, J=6.2 Hz, 3H, CH3-21), 2.98 (s, 3H, OMS), 3.66 (s, 3H), 4.46 (m, 1H, CH-3).

Methyl-3α-mesyloxy-12α-hydroxy-5β-cholane-24-oate (17): White solid; yield=83%; 1H NMR (CDCl3, 300 MHz): 0.65 (s, 3H, CH3-18), 0.90 (s, 3H, CH3-19), 0.93 (d, J=6.0 Hz, 3H, CH3-21), 3.00 (s, 3H, OMS), 3.66 (s, 3H), 3.94 (s, 1H, C-12) 4.60 (m, 1H, CH-3).

Methyl-3α-mesyloxy-7α, 12α-dihydroxy-5β-cholane-24-oate (18): White solid; yield=81%; 1H NMR (CDCl3, 300 MHz): 0.69 (s, 3H, CH3-18), 0.91 (s, 3H, CH3-19), 0.98 (d, J=6.2 Hz, 3H, CH3-21), 2.98 (s, 3H, OMS), 3.66 (s, 3H), 3.87 (m, 1H, C-12), 4.00 (m, 1H, C-7) 4.46 (m, 1H, CH-3).

Synthesis of methyl-3-azido-7α, 12α-dihydroxy-5β-cholane-24-oate (19), methyl-3-azido-12α-hydroxy-5β-cholane-24-oate (20), methyl-3-azido-7α, 12α-dihydroxy-5β-cholane-24-oate (21) [19,22]: To a solution of 4,5,6 (1 mmol) in dry CH2Cl2 (20 mL) was added triethylamine (0.1 mmol) at 0°C. Methane sulfonyl chloride, 1.0 equiv of N,N′-di-tert-butylcarbonyl) thiourea as a white solid (86% yield). Mp 127-129 °C, 1H NMR (CDCl3) 1.50 (brs, 18 H) (Figure 1).

General procedure for the synthesis of methyl-3β-Boc-protected guanidine-5β-cholane-24-oate (25), methyl-3β-Boc-protected guanidine-12α-hydroxy-5β-cholane-24-oate (26), methyl-3β-Boc-protected guanidine-7α,12α-dihydroxy-5β-cholane-24-oate (27) [21]: Each of the corresponding anilines was treated in DCM at 0 °C with 1.1 equiv of mercury (II) chloride, 1.0 equiv of N,N′-di-tert-butoxycarbonyl) thiourea and 3.1 equiv of TEA. The resulting mixture was stirred at 0°C for 1 h and for the appropriate duration at room temperature. Then, the reaction mixture was diluted with EtOAc and filtered through a pad of celite to give N,N′-Bis-tert-butoxycarbonylthioure (23). White solid; yield=88%; 1H NMR (CDCl3, 300 MHz): 0.69 (s, 3H, CH3-18), 0.98 (d, J=6.0 Hz, 3H, CH3-21), 3.65 (s, 3H), 3.86 (m, 1H, C-12), 3.98 (bs, 1H, C-7) 3.99 (s, 1H, CH-3); N,N′-Bis-tert-butoxycarbonylthioure (24).

To a stirred solution of thiourea (571 mg, 7.50 mmol) in THF (150 mL) under argon at 0 °C were added hexane and sodium hydride (1.35 g, 33.8 mol, 60% in mineral oil). After 5 min, the ice bath was removed, and the reaction mixture was stirred at room temperature for 10 min. The resulting slurry was stirred for another 2 h at room temperature. Then the reaction mixture was quenched with an aqueous solution of saturated NaHCO3 (10 mL). The reaction mixture was poured into water (250 mL) and extracted with EtOAc (3 x 70 mL). The combined organic layer was dried over Na2SO4, filtered, and concentrated in vacuo to give N,N′-Bis-tert-butoxycarbonylthioure (23) and N,N′-Bis-tert-butoxycarbonylthioure (24). The combined organic layer was stirred with cold water and brine. Solvent was evaporated under reduced pressure to afford crude products 19, 20 and 21 which was purified by column chromatography on silica gel (10% EtOAc/ hexane) to produce pure compounds 19, 20 and 21.

Synthesis of methyl-3-azido-7α, 12α-dihydroxy-5β-cholane-24-oate (19), methyl-3-azido-12α-hydroxy-5β-cholane-24-oate (20), methyl-3-azido-7α, 12α-dihydroxy-5β-cholane-24-oate (21) [19,22]: To a solution of 16,17,18 (1 mmol) in dry DMF (10 mL), sodium azide (5 mmol) was added triethylamine (0.1 mmol) at 0°C. Methane sulfonyl chloride, 1.0 equiv of N,N′-di-tert-butylcarbonyl) thiourea as a white solid (86% yield). Mp 127-129 °C, 1H NMR (CDCl3) 1.50 (brs, 18 H) (Figure 1).
**Designed and synthesized steroid carrier.**

a) CH$_3$OH, Acid, 10-15 h, RT  
b) Ethylene diamine, CH$_3$OH, Overnight, 50°C  
c) Boc Thiourea, HgCl$_2$, DCM, TEA, 3-5 h, 0°C-RT  
d) TFA: DCM(1:1), RT, 3 h, Resine Cl$^-$, 24 h, RT  
e) Methane sulfonyl chloride, DCM, 0°C  
f) NaN$_3$, DMF, 80-90°C, 10-15 h  
g) TPP, THF: H$_2$O(100:0.1), 50°C, 4-6 h  
h) Boc Thiourea, HgCl$_2$, DCM, TEA, 3-5hrs, 0°C-RT  
i) TFA: DCM(1:1), RT, 3 h.
Methyl-3β- Boc-protected guanidine-7α, 12α-dihydroxy-5β-cholane-24-oate (27). White solid; yield=76%; 1H NMR (CDCl 3, 500 MHz): 0.69 (s, 3H, CH 3-18), 0.92 (d, J=5.0 Hz, 3H, CH-21), 1.02 (s, 3H, CH 3-19), 1.35 (s, 3H, 1H, CH-3), 3.84 (s, 1H, CH-12), 3.98 (bs, 1H, CH-3) 4.41 (br s, 1H, CH-3); 13C NMR (126 MHz; CDCl 3): 11.89, 17.89, 20.83, 23.38, 24.32, 25.20, 25.75, 27.26, 27.72, 29.31, 31.56, 38.50, 39.65, 41.41, 41.90, 49.69, 54.30, 57.41, 57.79, 67.89, 72.58, 73.07, 140.29, 157.06 174.43.

Synthesis of N-Lithocholyl ethylamidine (7). N-deoxycholyl ethylamidine (8), N-cholyl ethylamidine (9): Compounds 4, 5 and 6 (10 g, 23.66 mmol) were treated with an excess of ethylamidine (15 ml) in methanol (50 ml). The reaction mixture was stirred at room temperature for 48 h, then poured over ice-cold water (400 ml); the solid reaction mixture was obtained was filtered off, dried, and purified by recrystallization from chloroform–methanol to get the pure products 7, 8 and 9.

Lithocholylethylamidine (7): White solid; yield=86%; 1H NMR (300 MHz, MeOD): 0.71(s, 3H, CH 3-18), 0.98 (s, 3H, CH-19), 1.01 (d, J=6.0 Hz, 3H, CH-21), 2.72 (t, J=6.3Hz 2H, NH CH 3), 3.24(t, J=6.3Hz 2H, NH CH 3), 3.57 (m, 1H, C-12) 3.95 (br s, 1H, CH-3).

Deoxycholylethylamidine (8). White solid; yield=85%; 1H NMR (300 MHz, MeOD): 0.70(s, 3H, CH 3-18), 0.93 (s, 3H, CH-19), 1.02 (d, J=6.3 Hz, 3H, CH-21), 2.70(t, J=6.3Hz 2H, NH CH 3), 3.22(t, J=6.3Hz 2H, NH CH 3), 3.57 (m, 1H, C-12) 3.95 (br s, 1H, CH-3).

Cholylethylamidine (9): White solid; yield=85%; 1H NMR (300 MHz, MeOD) 0.71(s, 3H, CH-18), 0.91(s, 3H, CH-19), 1.03 (d, J=6.3Hz, 3H, CH-21). 2.70(t, J=6.3Hz 2H, NH CH 3), 3.24-3.20(t, J=3.6Hz 2H, NH CH 3), 3.31 (m, 1H, C-12) 3.78(bs,1H, C-7) 3.95 (br s, 1H, CH-3).

General Procedure for the synthesis of Boc protected guanidine substituted at C-26 of lithocholyl ethyl amide (10). Boc protected guanidine substituted at C-26 of deoxycholyl ethyl amide (11), Boc protected guanidine substituted at C-26 of cholyl ethyl amide (12) [21]: Each of the corresponding anilines was treated in DCM at 0 °C with 1.1 equiv of mercury (II) chloride, 1.0 equiv of N,N′-di-(tert butyloxycarbonyl)imidazolidine-2-thione (for the 2-aminoimidazoline precursors), or N,N′-di-(tert-butyloxycarbonyl)thiourea (for the guanidine precursors) and 3.1 equiv of TEA. The resulting mixture was stirred at 0°C for 1 h and for the appropriate duration at room temperature. Then, the reaction mixture was diluted with EtOAc and filtered through a pad of celite to get rid of the mercury sulfide formed. The filter cake was washed with EtOAc. The organic phase was washed first with water (2 x 30 ml) and then with brine (1 x 30 ml), dried over anhydrous Na2SO4, and concentrated under vacuum to give a residue that was purified by neutral alumina column flash chromatography, eluting with the appropriate hexane: EtOAc mixture.

Boc protected guanidine substituted at C-26 of lithocholyl ethyl amide (10): White solid; yield=82%; 1H NMR (500 MHz; CDCl 3): 0.62(s, 3H, CH 3-18), 0.91 (br s, 6H, CH-19, CH-21), 1.50(s, 18H, Boc) 3.42(m, 2H, NH CH 3), 3.63(m, 3H, NHCH 3, CH-3), 7.29(s, 1H, CH-22-NH), 8.76(s, 1H, Boc-NH), 11.42(s, 1H, Boc-NH); 13C NMR (126 MHz; CDCl 3): 12.04, 18.40, 20.83, 23.38, 24.22, 26.43, 27.21, 28.03, 28.25, 30.56, 31.71, 33.56, 34.59, 35.36, 35.87, 36.49, 40.19, 40.44, 40.88, 42.12, 42.74, 56.14, 56.16, 71.86, 84.21, 152.88, 157.06 174.03.

Boc protected guanidine substituted at C-26 of deoxycholyl ethyl amide (11): White solid; yield=78%; 1H NMR (500 MHz; CDCl 3): 0.66(s, 3H, CH 3-18), 0.90 (s, 3H, CH-19), 0.98(d, J=6.0 Hz, CH-21), 1.50(s, 18H, Boc) 3.42(m, 2H, NH CH 3), 3.63(m, 3H, NHCH 3, CH-3), 7.41(s, 1H, CH-22-NH), 8.84(s, 1H, Boc-NH), 11.42(s, 1H, Boc-NH); 13C NMR (126 MHz; CDCl 3): 12.73, 17.45, 23.12, 23.65, 26.12, 27.12, 27.48, 28.00, 28.21, 28.55, 30.46, 31.57, 33.45, 33.62, 34.12, 35.26, 36.02, 36.42, 40.71, 42.08, 46.52, 47.33, 48.21, 71.79, 73.15, 84.16, 152.75, 156.96, 174.12.

Boc protected guanidine substituted at C-26 of cholyl ethyl amide (12): White solid; yield=77%; 1H NMR (500 MHz; CDCl 3): 0.65(s, 3H, CH 3-18), 0.87 (s, 3H, CH-19), 0.98(d, J=5.0 Hz, CH-21), 1.50(s, 18H, Boc) 3.38 (m, 3H, NHCH 3, CH-12), 3.53 (m, 3H, NHCH 3, CH-12), 3.97-3.97(m, 1H, C-3), 7.41(s, 1H, CH-22-NH), 8.84(s, 1H, Boc-NH), 11.42(s, 1H, Boc-NH); 13C NMR (126 MHz; CDCl 3): 12.88, 17.78, 22.05, 23.65, 26.12, 27.12, 27.48, 28.00, 28.21, 28.85, 30.46, 29.14, 29.81, 32.32, 34.72, 35.77, 40.19, 42.02, 41.16, 41.63, 46.38, 46.52, 47.33, 48.21, 68.41, 71.79, 73.03, 79.45, 83.46, 152.98, 157.24, 174.43.

General Procedure for the synthesis of Guanidine substituted at C-26 of lithocholyl ethyl amide (13). Guanidine substituted at C-26 of deoxycholyl ethyl amide (14), Guanidine substituted at C-26 of cholyl ethyl amide (15): Each of the corresponding Boc-protected precursors (0.5 mmol) was treated with 15 ml of a 50% solution of trifluoroacetic acid in DCM for 3 h. Then neutralize with ammonia.
solution and extracted with chloroform. The organic phase was washed first with water (2 × 30 mL) and then with brine (1 × 30 mL), dried over anhydrous Na2SO4, and concentrated under vacuum to give a residue that was purified by silica gel, eluting with the appropriate DCM: MeOH mixture.

Guanidine substituted at C-26 of lithocholy ethyl amide (13): White solid; yield=65%; 6H (500 MHz; CDCl3): 0.71 (s, 3H, CH3-18), 0.96 (br s, 3H, CH2-19), 0.98 (d, J=5.0, 3H, CH2-21), 3.1 (t, 2H, NH-CH3), 3.2 (t, 2H, NHCH3), 4.45 (m, CH-3); 13C NMR (126 MHz; MeOD): 11.06, 17.44, 20.53, 22.51, 23.85, 26.24, 26.94, 27.88, 28.65, 29.77, 31.87, 32.66, 34.26, 35.06, 35.46, 35.83, 35.95, 38.55, 40.12, 40.48, 42.12, 42.51, 56.01, 56.53, 70.99, 157.30, 175.79.

Guanidine substituted at C-26 of deoxycholy ethyl amide (14): White solid; yield=63%; 6H (500 MHz; CDCl3): 0.71 (s, 3H, CH3-18), 0.96 (s, 3H, CH2-19), 0.98 (d, J=5.0, 3H, CH2-21), 3.02 (m, 4H, NH2CH2), 3.56(m, 1H, C-12); 4.97(m, 1H, C-3 ); 13C NMR (126 MHz; MeOD): 11.06, 17.43, 20.52, 22.22, 23.83, 25.72, 26.06, 27.88, 29.77, 31.35, 31.72, 32.53, 34.26, 35.05, 35.83, 37.99, 39.97, 40.48, 41.80, 42.11, 42.49, 56.301, 56.53, 70.98, 79.34, 157.51, 176.41.

Guanidine substituted at C-26 of choly ethyl amide (15): White solid; yield=60%; 7H (500 MHz; CDCl3): 0.64(s, 3H, CH3-18), 0.85(s, 3H, CH2-19), 0.91(d, J=6.5, 3H, CH2-21), 3.3(m, 3H, NH2CH2CH2-12), 3.53(m, 2H, NHCH3), 3.82(br s, 1H, C-3); 4.97(m, 1H, C-3 ); 13C NMR (126 MHz; MeOD): 12.58, 18.07, 23.09, 24.22, 26.35, 26.89, 27.59, 28.22, 31.35, 31.00, 32.54, 32.86, 33.67, 34.94, 35.59, 35.80, 39.39, 42.08, 42.84, 43.32, 46.39, 50.87, 72.14, 80.12, 82.82, 158.93, 177.47. Sulfonyl chloride, DCM, TEA, 3-5Hrs, 0°C-RT

i) TFA: DCM (1:1), RT, 3 hrs.

II) TFA: DCM (1:1), RT, 3 hrs.

III) TFA: DCM (1:1), RT, 3 hrs.

IV) TFA: DCM (1:1), RT, 3 hrs.

V) TFA: DCM (1:1), RT, 3 hrs.

VI) TFA: DCM (1:1), RT, 3 hrs.

VII) TFA: DCM (1:1), RT, 3 hrs.

VIII) TFA: DCM (1:1), RT, 3 hrs.

IX) TFA: DCM (1:1), RT, 3 hrs.

X) TFA: DCM (1:1), RT, 3 hrs.

The stock solutions of the carriers samples were prepared for a specified time. After reaching the permeation time, the PAMPA sandwich (BD Gentest!, USA) was incubated for 5h without stirring [22-30].

**Influence of protein binding on carrier-siRNA nanoparticulate complexes:** Binding of drugs, specifically hydrophobic drugs to plasma proteins dramatically influence drug availability (fraction of free drug available for interaction for biological target is limited). Novel feature is connected with drug delivery using nanoparticles, where interaction of nanoparticles with plasma proteins has been for long time neglected. Recent findings gave sufficient evidence, that drug delivery using nanoparticles often exhibit reduced bioavailability by formation protein complexes on nanoparticle surfaces. In biological fluids, proteins bind to the surface of nanoparticles to form a coating known as the protein corona, which can critically affect the interaction of the nanoparticles with living systems. Addition of a protein corona (PC) or protein adsorption layer on the surface of nanomaterials following their introduction into physiological environments may modify their activity, bio-distribution, cellular uptake, clearance, and toxicity. Observed discrepancy between the in vitro and in vivo results for many nanoparticle based drug delivery systems is due, in part, to the adsorption of proteins and other biomolecules to the NP's surface upon exposure to the biological medium in vivo. The protein corona may also compromise the targeting efficiency of NPs that are functionalized with targeting ligands. In the other hand, protein corona for RNA nanoparticulate delivery system plays positive role, as was reported in literature. Our study describes initial formation of carrier-RNA complexes for stabilization of RNA and facilitated model transmembrane delivery with novel non-toxic steroid guanidine carriers. We formed initially carrier-RNA complex which upon addition of HSA forms ternary complex. This dynamic complex changes over the time over the time as we do expect that following mechanism: model

<table>
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<th>Carrier</th>
<th>C carrier·mmoll/l</th>
<th>C RNA·mmoll/l</th>
<th>nm</th>
<th>K ass</th>
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<td>1584</td>
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<td>14</td>
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<td>0.05</td>
<td>256</td>
<td>3311</td>
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**Table 1:** PAMPA conditions study of transport.
plasma protein (HAS) actually helps to release RNA from carrier-RNA complexes, as lipophilic carrier is transferred to hydrophobic domain of HSA and free RNA is slowly released over period of several hours as indicated from UV-Vis binding study. Our data are consistent with findings reported in recent literature for positive effects of RNA delivery influenced by plasma proteins [30,31].

**Transport efficiency and cytotoxicity of novel steroid carriers**

Primary leukemic cells (PLC-4T1) 200 μm were incubated for 2h with our novel transport agents C (0.1-1-10 mmol/l). Preliminary toxicity dates performed on several cell lines (PLC-4T1) showed no effect on the cell, which means our careers are not toxic and ready for in vitro and in vivo. The most efficient intracellular transport was detected with steroid carriers (Figures 3-5). RNA carrier application with will be tested in our laboratory (Figure 6).

**Conclusion**

While facilitated transport of DNA, RNA in vivo is based on
selective protein complexes, application of alternative ways is of great interest considering pharmaceutical potential of DNA/RNA fragment, mainly siRNA. We provide here synthetic carriers, designed such way that selective charge complementarity (guanidine-phosphate) and lipophilicity enhancement provided by steroid skeleton will synergically function as efficient polycationic carriers.

We present here series of steroid guanidine synthesis carries and testing of transport efficacy. In summary, we found that our novel synthetic carries indeed provide efficient transmembrane transport of RNA as was assessed by extensive PAMPA study.

In vivo transmembrane study along these lines are in progress in our laboratory.

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