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Enhanced transport of a novel anti-HIV agent—cosalane and its congeners across human intestinal epithelial (Caco-2) cell monolayers

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Abstract

Purpose: Cosalane is a potent inhibitor of HIV replication with activity against a broad range of viral targets. However, oral bioavailability of this highly lipophilic compound is extremely poor ($<1\%$). The purpose of this study is to screen a variety of permeation enhancers (cyclodextrin derivatives, cremophor EL, bile salts and mixed micelles) for their ability to enhance the transport of cosalane and its analogs/prodrugs across Caco-2 cell monolayers. *Methods:* Cosalane and its different analogs/prodrugs were synthesized and their physicochemical properties were determined. Caco-2 cells were cultured at a density of 66 000 cells/cm^2 either on collagen coated clear polyester membranes or Transwell® inserts. Side-bi-side diffusion cells and Transwell® inserts were employed to study for the transport of cosalane and its analogs/prodrugs with various permeation enhancers across Caco-2 cell monolayers. *Results:* Permeabilities of EH-3-39, EH-3-55 and EH-3-57 significantly improved compared to that of cosalane in the presence of bile salt, sodium desoxycholate. Among the various cyclodextrins studied, hydroxypropyl beta cyclodextrin (HP-β-CD) and dimethyl beta cyclodextrin (DM-β-CD) exhibited 22.3-fold and 19-fold permeability enhancement of cosalane respectively across Caco-2 cell monolayers. Sodium desoxycholate (10 mM) also showed a remarkable (105-fold) enhancement on the permeability of cosalane (P_{app} $11.72 \pm 3.31 \times 10^{-6}$ cm/s) without causing any measurable cellular damage. Cremophor EL resulted in higher transport of ^{14}C mannitol. The mechanism of enhancement effect can be mainly attributed to the alteration of membrane fluidity by cyclodextrin and opening of tight junctions by cremophor EL. *Conclusions:* Among the enhancers tested, 10 mM sodium desoxycholate and HP-β-CD appear to be viable candidates for further development of an oral formulation of cosalane and its congeners.

Keywords: Cosalane; HIV; Bile salts; Mixed micelles; Cyclodextrins; Caco-2 cell monolayers; Permeation enhancers

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1. Introduction

Cosalane (NSC 658586; Fig. 1) is a potent inhibitor of HIV replication with a broad range of activity against a variety of HIV-1 isolates, HIV-2, Rauscher murine leukemia virus, HSV-1, HSV-2, and human cytomegalovirus (Cushman et al., 1994, 1995; Golebiewski et al., 1996; Keyes et al., 1996; Casimiro-Garcia et al., 2001). It acts primarily by inhibiting the binding of gp 120 to CD4, as well as by inhibiting a post-attachment event prior to reverse transcriptase action (Cushman et al., 1994). The compound also inhibits activities of HIV-1 reverse transcriptase, protease, and integrase enzymes (Cushman et al., 1995; Keyes et al., 1996). The hydrophobic steroid moiety of cosalane appears to imbed perpendicularly in the lipid bilayer of the cell membrane and the viral envelope, with the dichlorodisalicylmethane fragment pointing outward in an obstructive mode (Cushman et al., 1995). Recently, it has been hypothesized that dichlorodisalicylmethane fragment, the 'pharmacophore' of cosalane, binds to both gp 120 and CD4, resulting in inhibition of gp 120 binding to CD4 (Cushman et al., 1998). Considering the advances in the crystal structure of CD4, it has been postulated that the most likely binding mode features two electrostatic interactions between the positively charged arginine 58 and arginine 59 residues of CD4 with the two negatively charged carboxylate residues of cosalane (Cushman et al., 1998).

Cosalane is a diprotic acid and is highly lipophilic (log octanol/water coefficient 6.8) (Venkatesh et al., 1996). A recent report from our laboratory pointed out that cosalane exhibited very low renal and biliary excretion in rats and accumulated to a significant extent in the liver, with very poor oral bioavailability ($<1\%$) (Kuchimanchi et al., 2000). Low oral bioavailability of cosalane was mainly attributed to its poor absorption across the enterocytes (Kuchimanchi et al., 2000). Cosalane is metabolically very stable with no metabolites detected in bile and urine following IV administration, or in liver and intestinal homogenate incubations (Udata et al., 1999). In a recent article, we reported that, the transcellular permeability (P_{app}) of cosalane across Caco-2 cell monolayers was extremely low (4.494×10^{-8} cm/s) and the effect of *p*-glycoprotein on the efflux of cosalane was not apparent (Pal et al., 2000). Also a characteristic disparity exists between the kinetics of cosalane uptake from apical (AP) donor solution and efflux into basolateral (BL) receiver side. Transport of cosalane across Caco-2 cell monolayers was extremely limited and appeared to be kinetically regulated by the equilibrium between the protein-bound and free drug partitioning into the cell membrane along with poor aqueous solubility (Pal et al., 2000). Cosalane mainly partitions and then sequesters into the lipid bilayers of the cell membrane, which probably limits its absorption through the enterocytes. In addition, it was proposed that cosalane may form micellar structure, which can hinder its absorption across intestine (Udata, 2000).

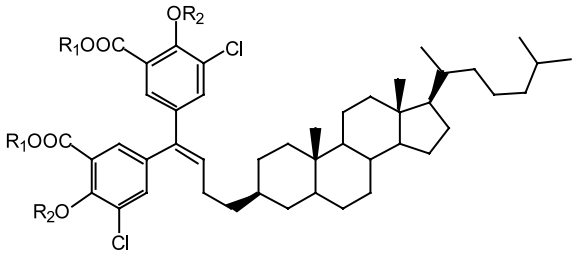

Fig. 1. Structural skeleton of cosalane and its congeners.

Absorption enhancers of varying origin have been employed previously to enhance the transepithelial transport of poorly absorbable drugs (Aungst, 1993; Ogiso et al., 1995). Absorption of water-insoluble compounds can often be increased with a variety of chemical enhancers, such as bile salts (Tengamnuay and Mitra, 1990), fatty acids (Shao and Mitra, 1992), bile salt–fatty acid mixed micelles (Poelma et al., 1990; Dangi et al., 1998) ionic and nonionic surfactants (Nerurkar et al., 1996), and cyclodextrin derivatives (Hovgaard and Brondsted, 1995). The use of cyclodextrins and bile salts/mixed micelles for improving the nasal delivery of peptides has also been studied extensively (Tengamnuay and Mitra, 1990; Shao and Mitra, 1992; Li et al., 1993). Since transport of cosalane across Caco-2 cell line is very limited and the mechanism of its transport is not well established, the present work deals with screening a

variety of permeation enhancers for their ability to enhance the transport of cosalane and its congeners across Caco-2 cell monolayers, a viable in vitro model for efficacy evaluation of absorption enhancers (Hovgaard and Brondsted, 1995).

2. Materials and methods

2.1. Materials

Hydroxy-propyl-β-cyclodextrin and dimethyl-β-cyclodextrin were received from Acros Organics (Fisher Scientific Co.), and Pharmatec Inc. Florida, respectively. Sodium taurocholate was purchased from Amerid Drug Chemical Company, NY HPLC-grade solvents methanol, tetrahydrofuran, phosphoric acid, acetonitrile were obtained from Fisher Scientific Company (Pittsburgh, PA). Cremophor EL, cyclodextrins (α, β and γ), sodium desoxycholate, sodium glycocholate, oleic acid, and all other chemicals, otherwise mentioned, were obtained from Sigma Chemical Company (St. Louis, MO).

2.1.1. Cell culture

Human colon carcinoma derived Caco-2 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). The growth medium, Dulbecco's modified Eagle Medium-F-12 was obtained from Life Technologies (Grand Island, NY). MEM non-essential amino acids (NEAA), penicillin, streptomycin, sodium bicarbonate, HEPES were purchased from Sigma Chemical Company (St. Louis, MO). Fetal bovine serum (FBS) was purchased from JRH Biosciences (Lenexa, KS). The buffer used in transport studies was Dulbecco's modified phosphate buffer saline (DPBS), containing 129 mM NaCl, 2.5 mM KCl, 7.4 mM Na_2HPO_4, 1.3 mM KH_2PO_4, 1 mM $CaCl_2$, 0.7 mM $MgSO_4$ and 5.3 mM glucose at pH 7.4. Culture flasks (75 cm^2 growth area), polyester Transwells® (pore size 3.0 μm with diameter 24.5 mm) and 13 mm clear polyester filters (3.0 μm pore size) were procured from Costar (Bedford, MA).

2.2. Methods

2.2.1. Cosalane di(methyl ester) (EH-5-40)

Cosalane (Fig. 1) was synthesized as previously described (Cushman et al., 1994). Cosalane (308 mg, 0.394 mmol) was dissolved under argon in a mixture of methylene chloride (0.8 ml) and *N*,*N*-diisopropylethylamine (0.2 ml, 1.1 mmol), and the mixture was cooled to −20 °C (carbon tetrachloride-dry ice bath) and stirred for 5 min. Methanol (0.2 ml) was injected and the reaction mixture was stirred for 25 min. BOP [benzotriazol-1-yl oxytris (dimethyl amino)-phosphonium hexafluorophpsphate] (352 mg, 0.8 mmol) was added in one portion to the solution and the mixture was stirred for 12 h under Ar with gradual warming to ambient temperature. The mixture was quenched with CH_2Cl_2 (2 ml) and washed with 0.1 N HCl (1 ml) and 10% $NaHCO_3$ -brine (2 drops in 1 ml). The organic layer was dried (Na_2SO_4) overnight. Solvents were evaporated and the residue purified by flash column chromatography on silica gel (14 g), eluting with hexane–ethyl acetate 20:1. Cosalane di(methyl ester) (152 mg, 48%) was isolated as a colorless solid: mp 50–54 °C. ^1H NMR ($CDCl_3$, 300 MHz) δ 11.36 (s, 1 H), 11.27 (s, 1 H), 7.52 (d, $J = 1.5$ Hz, 1 H), 7.49 (d, $J = 1.9$ Hz, 1 H), 7.38 (d, $J = 1.9$ Hz, 1 H), 7.32 (d, $J = 1.5$ Hz, 1 H), 5.95 (t, $J = 7.5$ Hz, 1 H), 3.95 (s, 3 H), 3.94 (s, 3 H), 2.06 (q, $J = 7.6$ Hz, 2 H), 1.92 (d, $J = 12.5$ Hz, 1 H), 1.75 (broad m, 1 H), 1.55–0.83 (m, 31 H), 0.87 (d, $J = 6.4$ Hz, 3 H), 0.84 (dd, $J = 6.7$ and 1.9 Hz, 6 H), 0.70 (s, 3 H), 0.62 (s, 3 H); IR (KBr) 3091 (broad band), 2928, 2858, 1682, 1451, 1330, 1244, 1181, 980/cm; FABMS *m/e* (rel intensity) 796.0 (M+2, 5.7), 795.0 (M+1, 10.4), 794.3 (M^+, 5.8), 765.0 (8), 763.0 (7),614.8 (26), 613.8 (17), 612.8 (64). Anal. Calcd for $C_{47}H_{64}Cl_2O_6$ C, 70.93; H, 8.10. Found: C, 71.30, H, 8.48.

2.2.2. O,O-Di(acetyl) cosalane (EH-3-39)

Acetyl chloride (0.26 ml, 3.7 mmol) was added dropwise to a solution of cosalane (307 mg, 0.4 mmol) and DMAP (5 mg, 0.04 mmol) in dry tetrahydrofuran (5 ml), and dry pyridine (0.3 ml, 3.7 mmol) was added. The mixture was stirred at ambient temperature for 3 days. The precipitate was filtered off and washed with THF (20 ml). The

residue, consisting of the bis (mixed anhydride), was dissolved in a mixture of THF (5 ml) and water (1 ml) and stirred with a sodium bicarbonate solution (10%, 1.5 ml) at ambient temperature for 75 min. The hydrolysis of the mixed anhydride was monitored by TLC. The mixture was acidified with 1 M HCl and evaporated. The residue was flash chromatographed on silica gel (15 g), eluting with hexane–ethyl acetate (3:1), followed by $CHCl_3$–THF–AcOH (300:60:1), to afford the product (170 mg, 47%) as a colorless, amorphous solid: ^{1}H NMR δ 7.77 (d, $J = 2$ Hz, 1 H), 7.68 (d, $J = 2$ Hz, 1 H), 7.52 (d, $J = 2$ Hz, 1 H), 7.47 (d, $J = 2$ Hz, 1 H), 6.14 (t, $J = 8$ Hz, 1 H), 2.38 (s, 3 H), 2.31 (s, 3 H), 2.17–0.85 (broad m, 39 H) 0.80 (d, $J = 8$ Hz, 6 H), 0.72 (s, 3 H), 0.60 (s, 3 H); IR (KBr) 2924, 1774, 1703, 1464, 1369, 1189, 1098, 890/cm. Anal. Calcd for $C_{49}H_{64}Cl_2O_8 \times 0.8$ AcOH: C, 67.53; H, 7.53. Found: C, 67.19; H, 7.89. The disodium salt was prepared was described below for EH-3-55: FABMS *m/z* (rel intensity) 920 (27), 919 (51), 918 (42), 917.2 ($M^+ + Na^+$, 80), 875.2 (98), 855.2 (45), 833.2 (89), 815.0 (100).

2.2.3. *O,O-Di(isobutyryl) cosalane (EH-3-55)*

Isobutyryl chloride (144 mg, 1.35 mmol) was added drop wise to a solution of cosalane (230.3 mg, 0.3 mmol) and 4-dimethylaminopyridine (4 mg, 0.03 mmol) in dry tetrahydrofuran (4 ml), followed by addition of dry pyridine (0.23 ml, 2.8 mmol). The mixture was stirred at ambient temperature for 4 days. The precipitate was filtered off and washed with THF (5 ml). The filtrate was evaporated to dryness. The residue was dissolved in methylene chloride (4 ml), washed with brine (0.5 ml) and the solvent was evaporated. The residue was dissolved in a mixture of THF (5 ml) and water (1 ml) and stirred with a sodium bicarbonate solution (10%, 1.5 ml) at ambient temperature for 4 h. Hydrolysis of this mixed anhydride was monitored by TLC. The mixture was acidified with 1 M HCl and evaporated. The residue was flash chromatographed on silica gel (15 g), eluting with hexane–ethyl acetate (3:1, 2:1), followed by ethyl acetate. Evaporation of the chromatography solvent afforded the product (95 mg, 35%) as an amorphous solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.78 (d, $J = 2$ Hz, 1 H), 7.70 (d, $J = 2$ Hz, 1 H), 7.51 (d, $J = 2$ Hz, 1 H), 7.48 (d, $J = 2$ Hz, 1 H), 6.16 (t, $J = 8$ Hz, 1 H), 2.91 (m, 1 H), 2.66 (m, 1 H), 2.12 (m, 1 H), 1.77–0.86 (m, 38 H), 1.38 (d, $J = 7$ Hz, 6 H), 1.35 (d, $J = 6$ Hz, 6 H), 0.91 (d, $J = 8$ Hz, 3 H), 0.88 (d, $J = 7$ Hz, 3 H), 0.72 (s, 3 H), 0.63 (s, 3 H); IR (KBr) 2930, 1774, 1703, 1464, 1260, 1086, 906/cm. To obtain the di(sodium salt), the diacid product was dissolved in tetrahydrofuran (2 ml). A saturated aqueous solution of $NaHCO_3$ (25 mg, 0.3 mmol) was added to the mixture. The precipitated inorganic salts were filtered off and solvents were evaporated. The disodium salt was obtained as a solid, amorphous residue after drying in vacuum for 48 h: IR (KBr) 3500 (broad band), 2920, 2862, 1746, 1631, 1567, 1468, 1372, 1259, 1094, 838, 745/cm; FABMS *m/e* (rel. intensity) 976 (41), 975 (75), 974 (61), 973 ($M + Na^+$, 96), 907 (25), 906 (33), 905 (58), 904 (51), 903 (82), 819 (36), 818 (43), 817 (73), 816 (50), 815.2 (100), 795 (40), 794 (36), 793.0 (59). Anal. Calcd for $C_{53}H_{70}Cl_2Na_2O_8 \times 4.4$ H_2O: C, 61.73; H, 7.70. Found: C, 61.88, H, 7.74.

2.2.4. *O,O-Di(pivaloyl) cosalane (EH-3-57)*

Pivaloyl chloride (163 mg, 1.35 mmol) was added drop wise to a solution of cosalane (230.3 mg, 0.3 mmol) and DMAP (4 mg, 0.03 mmol) in dry tetrahydrofuran (4 ml), followed by addition of dry pyridine (0.23 ml, 2.8 mmol). The mixture was stirred at ambient temperature for 4 days. The precipitate was filtered off and washed with THF (5 ml). The filtrate was evaporated to dryness. The residue was dissolved in methylene chloride (4 ml), washed with brine (0.5 ml), and the solvent was evaporated. The residue was dissolved in a mixture of THF (5 ml) and water (1 ml) and stirred with a sodium bicarbonate solution (10%, 1.5 ml) at ambient temperature for 3 h. The hydrolysis of the mixed anhydride was monitored by TLC. The mixture was acidified with 1 M HCl and evaporated. The residue was flash chromatographed on silica gel (15 g), eluting with hexane–ethyl acetate (3:1, 2:1), followed by ethyl acetate. Evaporation of the solvent afforded the diacid (120 mg, 42%) as an amorphous solid: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.77 (d, $J = 2$ Hz, 1 H), 7.72 (d, $J = 2$ Hz, 1 H), 7.46 (m, 2 H), 6.18 (t, $J = 8$ Hz, 1 H), 2.14 (m, 2 H), 1.77–0.86 (m, 32 H), 1.42 (s, 9 H), 1.21 (s, 9

H), 0.89 (d, $J = 7$ Hz, 2 H) 0.86 (d, $J = 7$ Hz, 6 H), 0.74 (s, 3 H), 0.66 (s, 3 H); IR (KBr) 2930, 1763, 1702, 1462, 1234, 1090, 895/cm. To obtain the di (sodium) salt, the diacid product was dissolved in tetrahydrofuran (2 ml). A saturated aqueous solution of $NaHCO_3$ (25 mg, 0.3 mmol) was added to the mixture. The precipitated inorganic salts were filtered off and solvents were evaporated. The disodium salt was obtained as a solid, amorphous residue after drying in vacuum for 48 h: IR (KBr) 3388 (broad band), 2925, 2862, 1744, 1608, 1566, 1460, 1376, 12803, 1131/cm; FABMS *m/e* (rel. intensity) 1004 (42.8), 1003 (76.8), 1002 (69.6), 1001.2 ($M+Na^+$, 100), 982 (12.5), 981 (16.1), 980 (12.5), 920 (12.5), 919 (21.4), 918 (32.1), 917 (39.2), 916 (38.3), 881 (10.7), 880 (12.5), 879 (30.4), 878 (21.4), 877 (35.5), 819 (12.5), 818 (16.1), 817 (42.8), 816 (28.5), 815 (55.4). Anal. Calcd for $C_{55}H_{74}Cl_2Na_2O_8 \times H_2O \times 1.5\ C_4H_8O$: C, 66.23; H, 8.02. Found: C, 66.50, H, 8.40.

2.3. General

Melting points were determined in capillary tubes on a Mel-Temp apparatus and are uncorrected. Spectra were obtained as follows: CI mass spectra on a Finnegan 4000 spectrometer, FAB mass spectra (FABMS) on a Kratos MS50 spectrometer, ^1H NMR spectra on a Bruker AC 300 spectrometer, and IR spectra on a Nicolet FT-IR Impact 410 spectrophotometer. Microanalyses were performed at the Purdue Microanalysis Laboratory.

2.4. Cell culture

Caco-2 cells (passages 19–30) were cultured in DMEM supplemented with 10% FBS (heat inactivated), 1% NEAA, 4 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 14 mM HEPES, pH 7.4. Cells were plated at a density of 66 000/cm^2 either on collagen coated clear polyester membranes (13 mm diameter, pore size 3 μm) placed on a tissue culture treated plastic dishes or on Transwell inserts. Caco-2 cells used for these experiments were incubated at 37 °C in humidified atmosphere of 5% CO_2/95% air, and were allowed to grow for 21–27 days. Trans-epithelial electrical resistance (TEER) and flux of ^{14}C-Mannitol were measured as markers cellular integrity. TEER values of cell monolayers were $> 300\ \Omega\ cm^2$ and ^{14}C-Mannitol transport was $< 0.5\%$ per hour in representative cell monolayers. Prior to each experiment, cell monolayers were rinsed 3×10 min with DPBS solution containing 129 mM NaCl, 2.5 mM KCl, 7.4 mM Na_2HPO_4, 1.3 mM KH_2PO_4, 1 mM $CaCl_2$, 0.74 mM $MgSO_4$ and 5.3 mM glucose, pH 7.4.

2.5. Preparation of formulations

All formulations were prepared in DPBS (pH 7.4). Stock solution of cosalane (10 mM) was prepared in 10% ethanol and diluted to 250 μM concentrations (donor) with different enhancer solutions i.e. bile salts and mixed micellar solutions in DPBS as indicated in Table 2. Formulations containing various concentrations of sodium desoxycholate, sodium taurocholate, sodium gly-

Table 1
Various functional groups included in the structural skeleton of cosalane that leads to respective cosalane congeners

Compound name	R_1	R_2	MW	P_{app} ($\times 10^{-6}$ cm/s)
Cosalane[a]	–Na	–H	814	11.72
EH-5-40[b]	–CH3	–H	796	0.005
EH-3-39[b]	–Na	–COCH3	832	31.96
EH-3-55[b]	–Na	$-COCH(CH_3)_2$	952	34.43
EH-3-57[b]	–Na	$-COCH(CH_3)_2$	980	22.23

Apparent permeability coefficients of cosalane and its congeners in the presence of 10 mM sodium desoxycholate.
[a] P_{app} for cosalane without bile salt is $0.112 \pm 0.008 \times 10^{-6}$ cm/s.
[b] The permeability of cosalane congeners without the bile salt was undetectable.

Table 2
Apparent permeability coefficients of cosalane, mannitol and diazepam (3-h studies) in the presence of various permeation enhancers

Enhancer	Concentration (mM)	Cosalane solubility (mg/ml)[a]	% Mannitol transported (for 3 h)[b]	% Diazepam transported (for 3 h)[b]	P_{app} (cm/s $\times 10^{-6}$) ($\pm$SD)[b]
Control	–	$7.12 \times 10^{-3} \pm 0.42$	1.2 ± 0.3	5.61 ± 0.72	0.112 ± 0.008
Sodium glycocholate	10	ND	>10	>20	1.103 ± 0.02
Sodium taurocholate	10	ND	>20	>25	3.486 ± 0.11
Sodium desoxycholate	5	9.67	2.9 ± 0.91	7.31 ± 0.39	1.24 ± 0.08
	10	9.48	2.9 ± 1.06	8.92 ± 0.97	11.72 ± 3.31
	20	12.18	3.8 ± 1.14	10.98 ± 2.31	18.57 ± 4.29
	30	12.22	>10	>20	28.91 ± 5.29
	40	11.47	>10	>20	53.81 ± 6.88
Sodium desoxycholate + oleic acid	10 + 5[c]	14.22	>10	>25	13.57 ± 3.35
Sodium desoxycholate + oleic acid	10 + 10[c]	ND	>20	>25	13.93 ± 2.88
Sodium desoxycholate + soya lecithin	10 + 0.5[c]	ND	>20	>25	19.58 ± 4.78
Cremophor EL	0.001[c]	ND	2.7 ± 0.18	5.41 ± 0.44	0.08 ± 0.005
	0.1[c]	ND	3.2 ± 0.38	6.22 ± 0.68	0.25 ± 0.009
	10.00[c]	ND	10 ± 0.91	10.17 ± 1.81	1.52 ± 0.011
Cyclodextrins					
α-	5[c]	ND	0.95 ± 0.081	4.31 ± 0.51	1.20 ± 0.17
β-	1.8[c]	ND	0.66 ± 0.12	5.20 ± 0.33	0.20 ± 0.009
γ-	5[c]	ND	1.01 ± 0.096	7.36 ± 0.54	0.18 ± 0.012
Dimethyl-β-	5[c]	ND	1.26 ± 0.11	6.92 ± 0.31	2.1 ± 0.2
Hydroxypropyl-β-	5[c]	ND	1.37 ± 0.16	9.02 ± 1.63	2.5 ± 0.24

Note: P_{app} of cosalane in presence of various cyclodextrins were determined using Transwell inserts. ND, not determined.
[a] In phosphate buffer pH 7.4.
[b] $n = 3$–5.
[c] Concentration in % w/v.

cocholate, and mixed micelle solution of sodium desoxycholate-soya lecithin employed in this study are summarized in Table 2. Mixed micellar solutions were prepared by the addition of either soya lecithin (0.5% w/v) or oleic acid (5 and 10% w/v) to 10 mM sodium desoxycholate in DPBS. All the solutions were equilibrated at 37 ± 0.5 °C prior to an experiment. These solutions were freshly prepared prior to each experiment.

2.6. Permeability studies

Side-bi-side™ diffusion cells and Transwell inserts were both employed to study the effect of permeation enhancer on cosalane transport across Caco-2 cell monolayers. Three milliliter formulation containing 250 μM cosalane were placed in the donor chamber and 3 ml DPBS containing 0.001% cremophor EL was placed in the receiving chamber to prevent any cosalane adsorption to diffusion cells or Transwells. At predetermined time intervals 40 μl sample was removed from receiver chamber and was replaced by an equal volume of DPBS. All samples were stored at −20 °C until further analysis. Cumulative amount of cosalane transported was plotted against time. Flux and apparent permeability (P_{app}) constants of cosalane were calculated according to following equation.

$$P_{app} = \frac{V\mathrm{d}C}{AC_0\mathrm{d}t}$$

The quantity $V(\mathrm{d}C/\mathrm{d}t)$ in the above equation represents the steady state rate of appearance of

cosalane in receiver chamber, A is the cross-sectional area, and C_0 is the initial donor concentration at time $t=0$. Similar experimental and data analysis procedures were adopted for the cosalane congeners.

2.7. Solubility studies

Solubility of cosalane was measured in various formulations using shake-flask method as described previously (Venkatesh et al., 1996). Briefly, excess amount of cosalane was added to 5 ml phosphate buffer (pH 7.4) containing various concentrations of bile salts and mixed micellar solutions in screw-capped siliconized glass vials. The vials were then agitated in a shaker bath at 37 °C for 48 h. The suspension was centrifuged at $10\,000 \times g$ for 20 min and the supernatant was analyzed for cosalane by HPLC.

2.8. HPLC analysis

The samples were analyzed according to a published method (Udata et al., 1999). A fluorescence detector (Schoeffel, model 970, McPherson, Chelmsford, MA) (λ_{ex} 230 nm; λ_{em} 450 filter) was used to quantitate cosalane and its analogs. The mobile phase [methanol: tetrahydrofuran: phosphoric acid (74:25:1% v/v)] was pumped at a flow rate of 1 ml/min through a C_{18} reversed phase column (Luna, 5 μm, 4.6 mm i.d. × 25 cm, Phenomenex, Torrance, CA) at ambient temperature.

2.9. Data analysis

Data points represent means of three experiments and the student's t-test was applied to evaluate the significance of difference observed.

3. Result and discussion

Previously we have reported that transport of cosalane across Caco-2 cell monolayers is extremely limited and is kinetically regulated by the equilibrium between the protein-bound and free drug partitioning into the cell membrane along with poor aqueous solubility of the compound (Pal et al., 2000). Since cosalane does not appear to be metabolized in the liver (Udata et al., 1999), limited intestinal permeability may be the primary reason for its poor oral bioavailability (Kuchimanchi et al., 2000). The permeability of very lipophilic molecules across cellular barriers is limited by several factors such as membrane partitioning, protein binding and aqueous solubility. Hence, in this study we have mainly focused on the effect of various permeation enhancers such as cyclodextrin derivatives, bile salts, mixed micelles, and cremophor EL on the permeability of cosalane and its congeners across Caco-2 cell monolayers. Effect of these chemicals on the integrity of Caco-2 cell monolayers was monitored by measuring the mannitol and diazepam fluxes, markers of paracellular and transcellular membrane integrity, respectively.

3.1. Chemistry

In order to understand the possible role of carboxylate moieties on the transport of cosalane, both carboxylic acid groups were converted to the corresponding methyl esters and permeability coefficients of both cosalane and its diester were determined (Table 1). Phenolic hydroxyl groups were also acetylated with various acid chlorides and the carboxylic acids converted to their sodium salts.

3.2. Solubility studies

As summarized in Table 2, the solubility of cosalane in phosphate buffer was found to be 7.12 μg/ml. However, cosalane solubility was significantly higher in micellar solutions of sodium desoxycholate and it reached a plateau above 20 mM of sodium desoxycholate (Table 2). Since the CMC value of sodium desoxycholate is 20 mM, the solubility of cosalane is significantly enhanced in sodium desoxycholate solution above its CMC value. This may be due to the fact that, the micelles might incorporate free drug in its hydrophobic core, and thereby increase the solubility of the compound.

3.3. Permeability enhancement of cosalane

3.3.1. Bile salts and mixed micelles

Fig. 2 illustrates the typical permeability profiles of cosalane in the presence of bile salts i.e., sodium desoxycholate, sodium taurocholate and sodium glycocholate. The respective permeability coefficients of cosalane are noted in Table 2. Sodium desoxycholate (10 mM) showed significant (105-fold) enhancement in the permeability of cosalane compared to other two bile salts. Hence, sodium desoxycholate was selected for further studies. Different molar concentrations of sodium desoxycholate were used to study its effect on cell integrity and cosalane permeability. Fig. 3 also shows permeability of cosalane across Caco-2 cell monolayers as a function of sodium desoxycholate concentration. Permeability of cosalane exhibited a gradual rise along with an increase in the bile salt concentration. The permeability increased appreciably (105-fold) in the presence of 10 mM sodium desoxycholate, which did not cause any measurable cell membrane disruption, as evidenced by less than 1% mannitol transport per hour (Table 2). The integrity of the cell monolayers was affected only above 20 mM bile salt concentration as evident by the fact that the percent mannitol transport was higher than 1% per hour (Table 2). Hence, 10 mM of bile salt was selected as an optimum concentration for permeability enhancement studies of various cosalane congeners. Effects of mixed micelles on cosalane permeability were then studied by adding soya lecithin (0.5% w/v) and oleic acid (5 and 10% w/v) to 10 mM bile salt. Although the permeability of cosalane increased in the presence of mixed micelles (Table 2), the mechanism may be entirely attributed to a decrease in the cell membrane integrity. In the control Caco-2 cell monolayers, percent mannitol and diazepam transport was 0.4 and 1.98/h (Table 2) respectively. Mannitol and diazepam transports in presence of 20 mM bile salt were significantly higher than 1 and 2% per hour, indicating disruption of cellular barrier. Fatty acids, glycerides, and many surfactants are known to enhance membrane permeability, including the intestinal epithelium (Anderberg et al., 1992; Dimitrijevic et al., 1997). At low concentrations, the surfactants are incorporated into the bilayers, which changes the physical properties of the cell membranes. When the lipid bilayers are saturated with surfactants, mixed micelles begin to form, resulting in removal of phospholipids from the cell membranes and thereby causing an increase in diazepam perme-

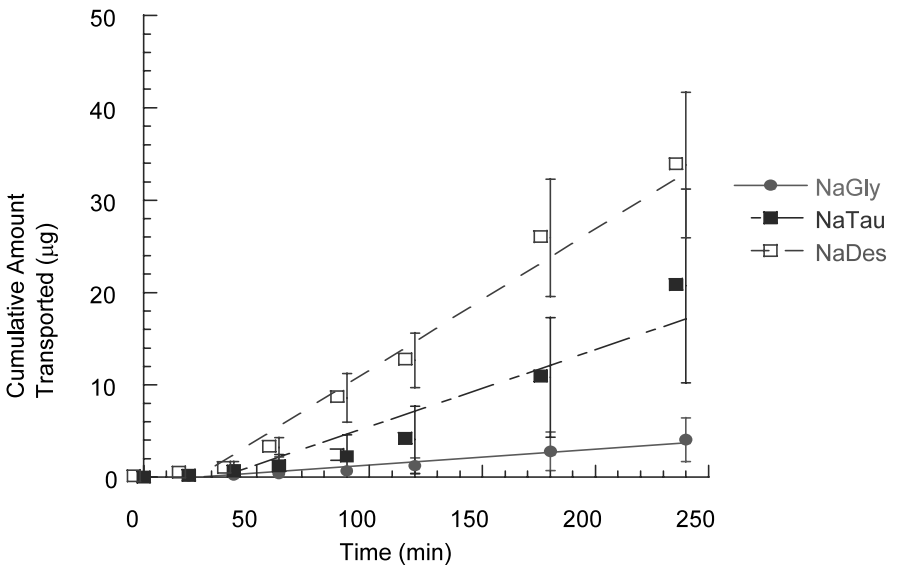

Fig. 2. Effect of bile salts (10 mM) on the transport of cosalane across Caco-2 cell monolayers.

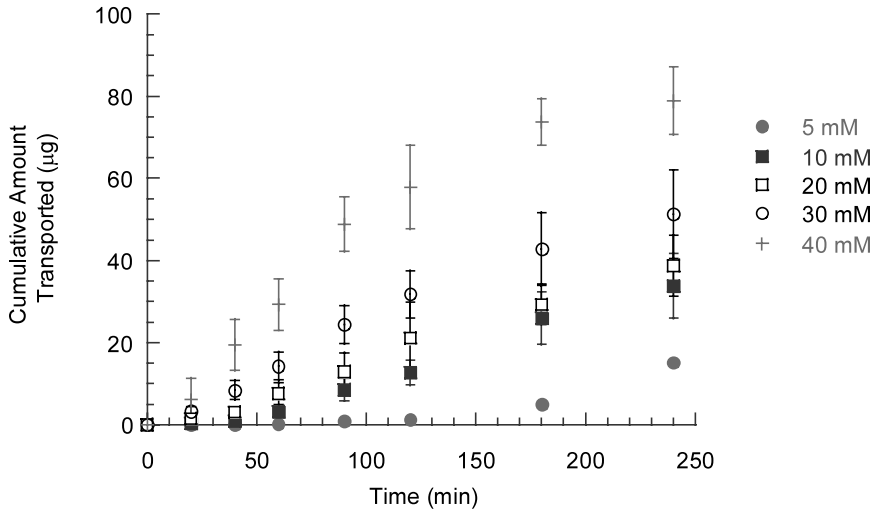

Fig. 3. Effect of various concentrations of sodium desoxycholate on the transport of cosalane across Caco-2 cell monolayers.

ability. When the micellar structures reach aqueous-membrane interface, the entrapped lipid molecules carried by the micelle may be absorbed into the mucosal cells. This process occurs by at least three different mechanisms: (1) the micelles might be taken up into the cell intact, (2) the constituent lipids might partition into the cell membrane during a direct interaction or 'collision' between the micelle and the cell membrane and (3) absorption might occur only through the monolayer phase of lipid molecules present in the aqueous environment in equilibrium with the lipids in the micelle (Westergaard and Dietschy, 1976).

Mixed micelles had a greater impact on the cellular integrity demonstrated by a significantly higher percent of mannitol transport (more than 1% per hour). Hence, mixed micelles may not serve as a physiologically acceptable permeation enhancer. Bile salts tend to dissolve the extra-cellular proteins and loosen the tight junctions (Freel et al., 1983). Moreover, these compounds may dissolve the membrane bound cholesterol and may increase the fluidity of the membrane, thereby increasing the transcellular permeability of compounds (Bakatselou et al., 1991). Since cosalane is a membrane interacting compound, any effect on the membrane fluidity would certainly result in the permeability change of this molecule.

Effect of 10 mM sodium desoxycholate on the transport of cosalane analogs/prodrugs was also studied and the results are summarized in Table 1. Various cosalane congeners were synthesized in order to increase the anti-HIV potency and to improve the oral bioavailability of cosalane. These congeners have similar anti-HIV potency as cosalane when tested in vitro, and therefore may serve as good candidates for the treatment of HIV infection. Interestingly, like cosalane itself, the permeability of cosalane congeners is negligible in the absence of permeation enhancers. In addition, the amino acids (diglycine and diaspertic acid) conjugates of cosalane failed to utilize the amino acid transporter and enhance cosalane permeability across intestinal mucosa (Kuchimanchi et al., 2002). In fact, transport across Caco-2 monolayers is essentially negligible by diester modification of this molecule (P_{app} 0.005, Table 1). Conversely, esterification of the phenolic hydroxyl groups of cosalane with various acid chlorides, results in a more lipophilic compound that facilitates transport. Moreover, the permeabilities of EH-3-39, EH-3-55 and EH-3-57 were significantly elevated compared to cosalane in the presence of bile salt, sodium desoxycholate. Appearance of these congeners across Caco-2 monolayers was undetectable without the bile salt. A combination of formulation approach and chemi-

cal structure modification appears to produce synergistic enhancement in Caco-2 permeability.

3.3.2. Surfactants (cremophor EL)

Cremophor EL is a surfactant and has been used successfully as a solubilizer in the formulations of cyclosporine A and taxol. Interestingly, cremophor EL did not have any significant effect on the permeability of cosalane across Caco-2 monolayers (Table 2). Cremophor EL may also enhance the intestinal absorption of drugs by inhibiting the apically polarized efflux system. However, cosalane neither is a substrate for *P*-glycoprotein nor does have dissolution rate-limited transport. Therefore, a negligible effect on the permeability of cosalane across Caco-2 cell monolayers by cremophor EL has been observed.

3.3.3. Cyclodextrins

Cyclodextrins are known to enhance the absorption of poorly water-soluble compounds by increasing the rate of dissolution as well as affecting the membrane structure (Gerloczy et al., 1994). The most common CD derivatives employed in pharmaceutical research are the dimethylated and hydroxypropylated beta-cyclodextrin (DM-β-CD, and HP-β-CD) (Rajewski and Stella, 1996). These CDs have higher aqueous solubility as well as a more hydrophobic cavity compared to the parent compound. Therefore, we studied the effect of various CDs on the transport of cosalane across Caco-2 cell lines and the results are summarized in Table 2. The CD concentration was maintained at 5%, except β-CD (1.8%) due to its limited solubility in transport buffer (DPBS). In contrast to bile salts, only HP-β-CD promoted diazepam (3% per hour) transport and none of the cyclodextrins affected mannitol transport across Caco-2 cell monolayers indicating minimal disruption of cellular barrier. Among the different CD-derivatives studied, DM-β-CD and HP-β-CD had significant enhancement ($P < 0.05$) effects on the transcellular permeability of cosalane. β-CDs solubilize steroids through the formation of inclusion complexes (Hovgaard and Brondsted, 1995; Rajewski and Stella, 1996). Since the entry and exit of steroid from the cavity remains unaffected, the compound is freely available for absorption. The success of CDs in raising oral bioavailability of drugs is well known, particularly when the rate-limiting step in drug absorption is poor dissolution (Hovgaard and Brondsted, 1995). Although β and γ-CDs did not appear to produce enhancement of cosalane transport, treatment of Caco-2 cells with DM-β-CD or HP-β-CD resulted in higher diazepam transport indicating alteration in membrane fluidity. Uekama et al. (1993) suggested that CDs might affect drug absorption through modification of the mucosal membrane. Free CDs tend to remove membrane components, therefore modifying transport properties of the membrane and facilitate drug absorption. This hypothesis is supported by higher diazepam transport in response to CDs (Table 2). Thus, it is reasonable to conclude that CDs may cause alterations the transport of cosalane based on their capacity to alter membrane fluidity. HP-β-CD caused almost 2-fold increase in diazepam transport and 20-fold increase in cosalane transport and therefore, could be a promising candidate for further evaluation. The absorption rate and bioavailability of lipophilic compounds can often be increased with surfactants as additives (Scott-Moncrieff et al., 1994).

In conclusion, the permeability of cosalane and its congeners across Caco-2 cell monolayer is extremely low in the absence of permeation enhancers. Among the enhancers tested, sodium desoxycholate at 10 mM concentration and HP-β-CD appear to be viable candidates for further development of an oral formulation of cosalane and its congeners. The exact mechanism of enhanced cosalane permeation in presence of sodium desoxycholate needs further investigation. Bile salts are known to form reverse micelles in the lipid bilayers with hydrophobic surface aligning with the lipid surface. These micelles may form a channel, which can displace bound cosalane from the lipid-sequestered sites allowing it to permeate from the AP to the BL side. Detailed in vivo/in situ studies are underway to further examine the suitability of sodium desoxycholate as an oral absorption enhancer of cosalane.

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