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Intestinal absorption and biodistribution of cosalane and its amino acid conjugates: novel *anti*-HIV agents

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Abstract

Cosalane and its amino acid conjugates are potent inhibitors of HIV replication. The purpose of this study was to investigate: (1) the pharmacokinetic disposition of the diglycine (GC) and the diaspartic acid (ASPC) conjugates of cosalane in male Sprague–Dawley rats; (2) intestinal absorption of cosalane and its amino acid conjugates using in vitro (small intestinal segments), in situ (closed loop); and (3) biodistribution of GC and its absolute oral bioavailability in rat. Cosalane and its conjugates exhibited biexponential disposition with very long half-lives upon intravenous dosing. However, these compounds failed to permeate the small intestine unless sodium desoxycholate (5–20 mM) was used as an intestinal permeation enhancer. A rank order correlation in terms of permeation enhancement in a descending order is as follows: GC > Cosalane > ASPC. In situ studies revealed that although the bile salt enhanced the permeation of cosalane across the enterocyte, its hepatic uptake was extensive. However, 66% of the absorbed dose of GC escaped uptake by the reticuloendothelial system (RES) and its biodistribution studies showed that the uptake by the RES was significantly lower compared to the parent compound. GC had an absolute oral bioavailability of $5.10 \pm 1.51\%$. Therefore, GC appears to be a favorable candidate for further development.

Keywords: Cosalane; Amino acid conjugates; Absorption enhancement; Intestinal transport; Closed loop method; Biodistribution

1. Introduction

In an intensified effort to improve the intestinal permeation of cosalane (Fig. 1A), its amino acid conjugates were synthesized (Santhosh et al., 2000). Although the carbonyl groups of cosalane are important for *anti*-HIV activity, it has been hypothesized that the two carboxyl groups of cosalane (Fig. 1B and C) could be modified via conjugation to amino acids without significantly compromising the *anti*-HIV activity since free carboxyl groups are afforded by the introduction of

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the amino acid residues. Such a modification led to the synthesis of the diglycine and diaspartic acid conjugates of cosalane. In addition, these two amino acid conjugates might target the gastrointestinal transporters involved in the absorption of amino acids and small peptides and thereby result in improved oral bioavailability.

Previous studies on the pharmacokinetic disposition of cosalane and dihydrocosalane revealed that the oral bioavailabilities of both compounds were extremely low ($< 1\%$) (Kuchimanchi et al., 2000). The primary reason for such low oral bioavailability may be attributed to the poor enterocytic permeation of these compounds (Pal et al., 2000). In order to improve the transport characteristics of cosalane, two amino acid conjugates were synthesized and evaluated for their *anti*-HIV activity. The diglycine (GC) and the diaspartic acid (ASPC) conjugates of cosalane exhibited good in vitro *anti*-HIV activities (EC_{50}: 5.1, 3.4 and 10.7 μM for cosalane, GC and ASPC, respectively, versus HIV-1_{RF} in CEM-SS cells) (Santhosh et al., 2000).

In vitro studies using excised rat small intestinal segments were performed to assess the permeability of cosalane, GC and ASPC. The tissue integrity was evaluated by measuring the transport of a paracellular (^{14}C-mannitol) and transcellular marker (^{14}C-diazepam). In situ studies were conducted in order to assess the extent of intestinal absorption and hepatic uptake of cosalane, GC and ASPC. Portal and jugular vein cannulated Sprague–Dawley rats were used in these studies.

In addition, the effect of various concentrations of bile salt–sodium desoxycholate (5–40 mM) on the absorption characteristics of these compounds was studied. Based on the in vitro and in situ absorption results, the compound showing the greatest promise in terms of intestinal permeation and decreased hepatic uptake (GC) was selected and its oral bioavailability and tissue distribution evaluated in the SD rat.

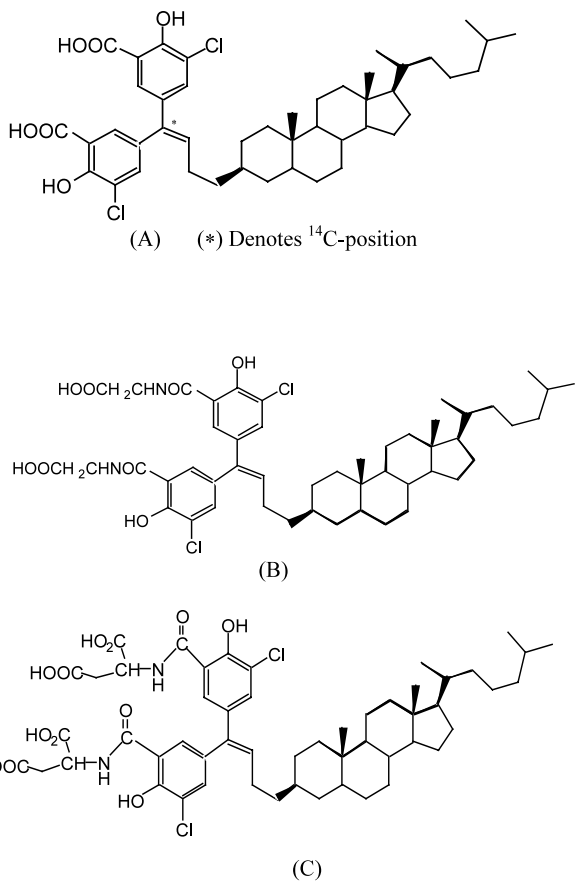

Fig. 1. Structures of: (A) cosalane; (B) diglycine conjugate of cosalane; and (C) diaspartic acid conjugate of cosalane.

2. Materials and methods

2.1. Materials

Cosalane disodium salt [1,1-di (3′-sodiumcarboxy-5′-chloro-4′-hydroxyphenyl)-4(3β-cholestanyl-1-butene], diglycine conjugate {5α-3β-[4,4-(5′, 5″-bis-*N*-(carboxymethyl)carbamoyl, 3′3″-dichloro-4′,4″-dihydroxy)-diphenyl-buten-3-yl]cholestane} and diaspartic acid conjugate {5α-3β-[4,4-(5′,5″-bis-*N*-(1,2 dicarboxymethyl)carbamoyl-3′3″-dichloro-4′,4″-dihydroxy)-diphenyl-buten-3-yl]cholestane} were kindly supplied by Professor Mark Cushman, Purdue University, West Lafayette, IN. HPLC-grade solvents used for HPLC analysis and sodium desoxycholate were obtained from Fisher Scientific Co. (St. Louis, MO). Nembutal sodium (sodium pentobarbital) was purchased from Abbott Laboratories (North Chicago, IL).

Ketaset (ketamine HCl) was obtained from Fort Dodge Animal Health (Fort Dodge, IA). Rompun (xylazine) was purchased from Bayer Corporation (Agricultural Division, Animal Health, Shawnee Mission, KS). All other chemicals were of analytical grade and were used as received.

2.2. Synthesis of cosalane and its amino acid conjugates

Cosalane was synthesized by following the reported procedure (Cushman et al., 1994). Cosalane was coupled with glycine *tert*-butyl ester hydrochloride in the presence of BOP and Et_3N in THF. Deprotection of the resulting intermediate by TFA gave the di-glycine conjugate. L-Aspartic acid di-*tert*-butyl ester hydrochloride was reacted with cosalane under similar conditions, followed by deprotection with TFA, to afford the di-aspartic acid conjugate of cosalane (Santhosh et al., 2000; Santhosh et al., in press).

2.3. Animals

Portal and/or jugular vein cannulated male SD rats weighing 200–250 g were obtained from Charles River (NY). Portal and jugular vein cannulated animals were used in the in situ studies while jugular vein cannulated rats were employed in intravenous, oral, and tissue distribution studies of GC. Whole animals without any surgical manipulation were employed in the in vitro transport studies.

2.4. In vitro, in situ, and in vivo studies

Transport studies were carried out in a Kreb's-Bicarbonate Ringer's solution (KBR) at a pH of 7.4. Sprague–Dawley rats (200–250 g) were used in accordance with the protocol approved by the University of Missouri-Kansas City. The rats were anesthetized by an intraperitoneal injection (1 ml/kg) with a mixture of Ketamine (100 mg/ml) and Xylazine (100 mg/ml) in the ratio of 9:1 v/v. Once the animals were under anesthesia, they were placed on a warming pad under a surgical lamp to maintain body temperature. Upon verification of loss of pain reflex, a midline incision of 3–4 cm was made and the small intestine was located. In the preliminary experiments, the small intestine was rapidly removed from the anesthetized rat and cut into the following segments: duodenum, jejunum and ileum. The sections were then washed in ice cold KBR solution and placed in beakers with KBR solution. The intestinal segments were allowed to equilibrate for approximately 30 min to minimize tissue damage during preparation. During the process of tissue preparation, the intestinal segments were left immersed in KBR solution, and pieces of approximately 1.0–1.5 cm were cut by opening the respective segments along their mesenteric border using blunt dissection. Small intestinal segments with Peyers patches were excluded from the study. In subsequent studies employing sodium desoxycholate, the ileal portion of the small intestine was used to evaluate the transport characteristics of GC and ASPC.

Following the completion of the tissue preparation, the segments were mounted in the side-by-side diffusion chambers (Crown Glass Company, NY) as flat sheets with a surface area of 0.636 cm^2. The chambers were maintained at 37 °C by water jackets. Three milliliter of KBR was added to each compartment of the diffusion chamber and the contents were stirred continuously.

At the start of an experiment, the KBR in the each of the chambers was replaced with 3 ml of preheated KBR to avoid temperature differences. Drug (200 μM) or the marker solutions (3.33 and 0.3 μCi/ml of [^{14}C]-diazepam and [^{14}C]-mannitol, respectively) with or without the enhancers were prepared in prewarmed KBR solution (37 °C) and placed in the donor chamber prior to the initiation of transport experiments. Aliquots (50 μl) were withdrawn from the receiver chamber at predetermined time intervals and were subjected to either HPLC analysis (amino acid conjugate and cosalane) or scintillation counting.

The absorption of cosalane and its amino acid conjugates in the presence and absence of sodium desoxycholate was examined by the in situ closed loop method (Nakanishi et al., 1989). Sprague–Dawley (200–250 g) rats were fasted overnight (12–18 h) with free access to water. In the in situ

studies, different segments (duodenum or jejunum or ileum), (10–15 cm) of the rat's small intestine were isolated. The intestinal contents were expelled from the respective segments and the segments were flushed with prewarmed (37 °C) isotonic phosphate buffered saline. Following this procedure, 1.5 ml of drug solution (1.2 mM) in isotonic phosphate buffered saline was introduced into the respective regions and the ends of the segments were tied with a silk suture. At various intervals of up to 3 h, blood samples (100 μl) were withdrawn from the portal and the jugular veins and analyzed for both cosalane and its amino acid conjugate(s).

For intravenous studies, GC and ASPC (1.0 mg/kg) solubilized in 20% DMSO was administered via the jugular vein as a bolus dose. Blood samples (100 μl) were collected at predetermined time points over a period of 72 h. Plasma was separated from blood samples and stored at −80 °C until further analyses.

The oral absorption study of GC (20.0 mg/kg) was carried out using 20 mM sodium desoxycholate in isotonic phosphate buffered saline (pH 7.4; 50 mM). Animals were fasted overnight (12–18 h) with free access to water. The formulation was administered by oral gavage (0.75 ml). Blood samples (100 μl) were collected from the jugular vein over a period of 24 h. For the in situ and in vivo studies, heparinized saline (100 μl) was injected through either the portal or jugular cannula following blood withdrawal in order to maintain a constant blood volume. Plasma was immediately separated by centrifugation and then stored at −80 °C until further analysis.

2.5. Tissue distribution

Two sets of tissue distribution studies were performed. In the first set, animals (three) were dosed with GC or cosalane intravenously (10 mg/kg in 20% DMSO). The animals were sacrificed by an overdose (1 ml/kg) of pentobarbital (100 mg/ml) at 2 h postdose. In the second set of studies, a solution of the non-essential amino acid glycine in IPBS (1.5 ml, 300 mM) was infused through the jugular vein over a period of 1.5 h at a slow rate with an infusion pump (CMA/102 microdialysis pump, CMA/Microdialysis, Acton, MA), which was followed by a bolus injection of cosalane (10 mg/kg in 20% DMSO). The animals were euthanized by an overdose of sodium pentobarbital as described above. Various tissues such as plasma, liver, spleen, lung, heart, kidney, and muscle were isolated 2 h postdose and were immediately stored at −80 °C until further analysis.

2.6. Plasma stability studies

In order to evaluate the stability of the amino acid conjugates in plasma, both GC and ASPC were incubated in rat plasma. Plasma (990 μl) was spiked with 2 mg/ml concentration (10 μl) of the amino acid conjugates ($n = 3$). The preparations were incubated in a 37 °C shaking water bath (Precision, MA). At predetermined time intervals of up to 10 h, 50 μl fractions were removed from the water bath and quenched with 450 μl volume of a mixture of acetonitrile and methanol (4:5 v/v). The samples were vortexed and subjected to centrifugation at 16,000 × *g* for 15 min at 4 °C (GS-15R centrifuge, Beckman). The supernatant was separated and 75 μl was injected onto the HPLC column. The % drug remaining with time was estimated.

2.7. Extraction procedures

Extraction studies with GC and ASPC showed that a mixture of acetonitrile and methanol in the ratio of 5:4 v/v was an excellent extraction medium generating a high efficiency and reproducibility for GC, ASPC and cosalane. Plasma samples or tissues (100 μl or 100 mg) were homogenized with a mixture of methanol and acetonitrile (5:4) (900 μl), vortexed and centrifuged (GS-15R centrifuge, Beckman) at 16,000 × *g* for 15 min at 4 °C. The supernatant was separated and 75 μl was injected onto the HPLC column. If the concentration was below the limit of detection (0.02 μg/ml), the supernatant was dried under vacuo in a speed vac system (ThermoQuest, OH) and the residue was reconstituted in 100 μl of methanol and acetonitrile mixture (5:4) before HPLC analysis.

2.8. Method development

In order to estimate the concentrations of GC, ASPC, and cosalane from in vitro, in situ, and in vivo studies, a suitable HPLC analytical technique has been developed. In order to estimate the parent compound cosalane, along with the amino acid conjugates, an isocratic method with tetrahydrofuran:methanol:phosphoric acid in the ratio of 14:85:1 for GC or 19:80:1 for ASPC was utilized.

2.9. Instrumentation

A Varian star HPLC system (Varian Inc., WalnutCreek, CA) consisting of Varian 9012 solvent module, 9095 autosampler, 9050 UV–Vis detector (λ_{max} 230 nm) connected in series with a Schoeffel 970 fluorescence detector (McPherson, Chelmsford, MA) attached to a Hewlett-Packard 33958 integrator was used for analysis of cosalane and dihydrocosalane. The excitation wavelength was 230 nm and emission wavelength was set at 450 nm. The mobile phase consisted of methanol: tetrahydrofuran:phosphoric acid with the composition described previously. The plasma samples were diluted with a mixture of methanol and acetonitrile (5:4 v/v). The samples were then subjected to centrifugation at 13,375 × *g* for 15 min at room temperature. The supernatant was collected and 75 μl injected on to a Macherey-Nagel C18 column (5 μm, 4.6 mm i.d. × 25 cm) after appropriate dilution.

2.10. Statistical analysis

Statistical analyses of the experimental data were performed using a Student's *t*-test and one way analysis of variance. Statistical difference was deemed significant at $P < 0.05$.

3. Results

3.1. Analytical procedures

The stabilities of GC and ASPC in a mobile phase containing phosphoric acid (1% v/v) were evaluated. The HPLC method was suitable for simultaneous analysis of both the amino acid conjugates and cosalane. Fig. 2 shows the chromatograms of GC (B) overlaid over those of cosalane (A) and a mixture of cosalane + GC (C), all obtained under identical chromatographic conditions. GC elutes at 9.3 min and is not hydrolyzed to cosalane in the presence of phosphoric acid. Cosalane (A) elutes as a broad peak around 22.5 min, while a good separation between the amino acid conjugate and cosalane (C) was achieved.

3.2. Plasma stability studies

Both GC and ASPC were stable in incubations with plasma over a period of 10 h at 37 °C. A plot of % remaining of GC and ASPC versus time (Fig. 3) indicates that neither of these conjugates were enzymatically hydrolyzed to any appreciable extent in plasma to cosalane.

3.3. Effect of sodium desoxycholate on the intestinal transport of amino acid conjugates

Fig. 4 shows the in vitro transport of the GC in the presence of 10 and 20 mM absorption enhancer, sodium desoxycholate. A comparison of the apparent permeabilities of GC in the presence of 10 mM (5.24×10^{-8} cm/s) and 20 mM (5.05×10^{-5} cm/s) desoxycholate showed that the higher concentration (20 mM) caused approximately 1000 fold enhancement in the GC absorption across the intestinal segment. The inset in Fig. 4 depicts the transport of GC in the presence of 10 mM enhancer across the intestinal segment. The inset in Fig. 5 shows the transport of ASPC in 20 mM desoxycholate formulation. A comparison of the in vitro transport profiles of ASPC and GC in the presence of 20 mM desoxycholate indicated that the transport of ASPC is approximately 60 fold lower compared to GC (Fig. 5 and Table 2). However, cosalane could not be detected either in the donor or the receiver chambers for the period of the experiment.

In order to assess the influence of desoxycholate on the tissue integrity, both transcellular (^{14}C-diazepam) and paracellular (^{14}C-mannitol) markers' permeability has been assessed. The tissue in-

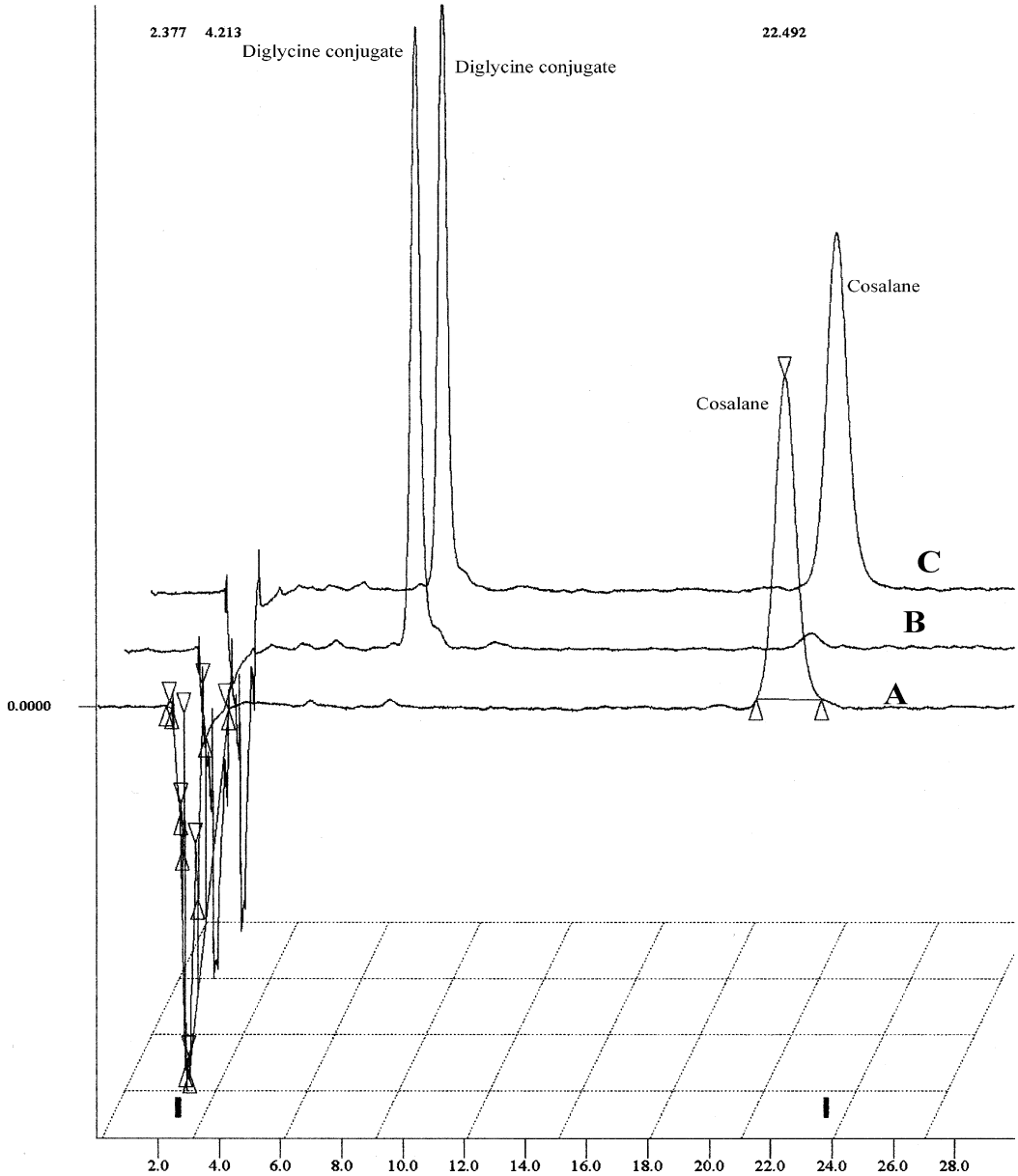

Fig. 2. Representative chromatograms showing diglycine conjugate and cosalane.

tegrity was lost beyond 2.5 h of exposure to the enhancer (20 mM) and the percent transport of diazepam increased by more than one order of magnitude (Table 1). In addition, the percent transport of ^{14}C-mannitol was significantly higher at all the concentrations of the enhancer employed (Table 1). The apparent permeability values of cosalane and its amino acid conjugates in the presence of 20 mM desoxycholate have been summarized in Table 2. The P_{app} of GC is >100 fold higher compared to both cosalane and ASPC.

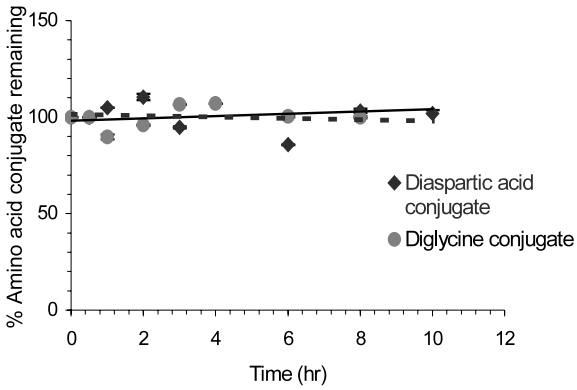

Fig. 3. Plasma stability study of amino acid conjugates of cosalane; ($n = 4$).

Cosalane absorption studies employing 10 and 20 mM enhancer (Fig. 6) were conducted in portal and jugular vein cannulated rats. Although increasing the enhancer concentration resulted in higher portal AUC values, systemic (plasma) levels of cosalane could not be observed from either of the formulations. However, similar absorption studies with the diglycine conjugate of cosalane revealed measurable portal and jugular GC concentrations. Approximately, 66% of the absorbed dose of GC was found in the systemic circulation (Fig. 7). However, measurable plasma concentrations of ASPC or cosalane were not detected either in the portal or in the jugular blood samples. In order to evaluate the tissue viability in the presence of desoxycholate, serum LDH activity was measured during the in situ studies. Serum LDH levels increased significantly up to almost 240% of the control values (Fig. 8), during the study period.

In addition, tissue distribution studies of cosalane before and after the infusion of glycine, and GC were conducted (Fig. 9). The reticuloendothelial (liver and spleen) uptake of cosalane postglycine infusion, and its amino acid conjugate-GC were reduced by more than 50% compared to that of cosalane (Fig. 9).

Intravenous studies of cosalane, GC and ASPC showed that all these compounds exhibit a biex-

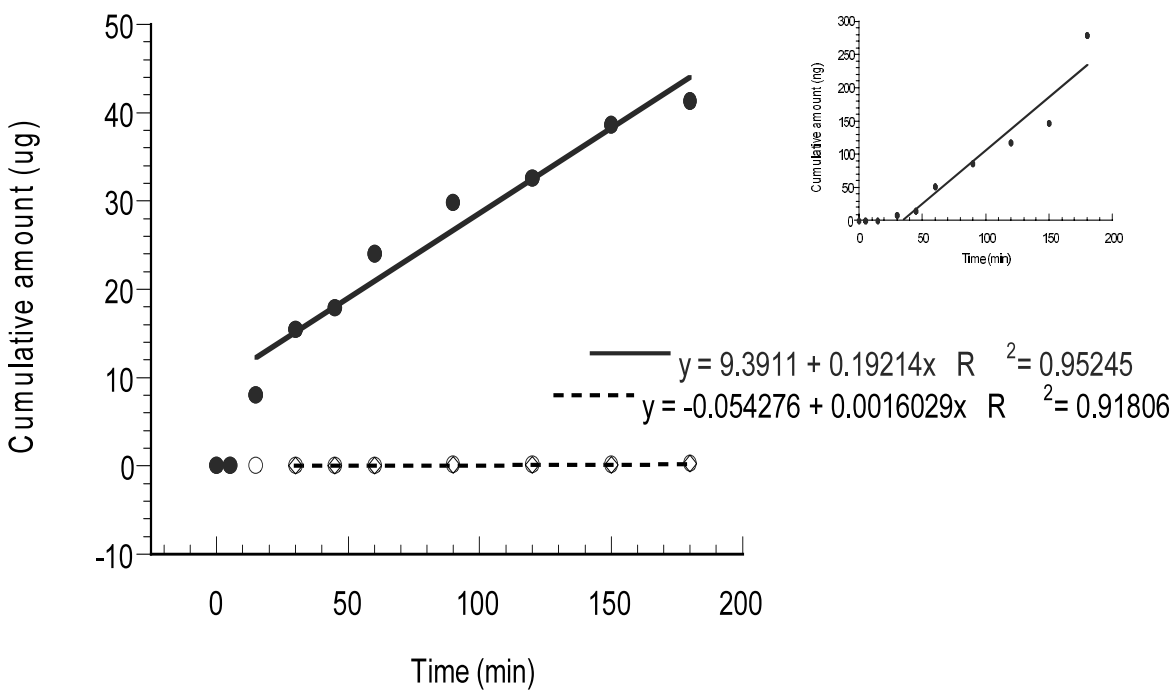

Fig. 4. In vitro transport of diglycine conjugate of cosalane in the presence of 10 (open diamond) and 20 mM (closed circle) sodium desoxycholate across SD rat small intestinal segment; ($n = 3$).

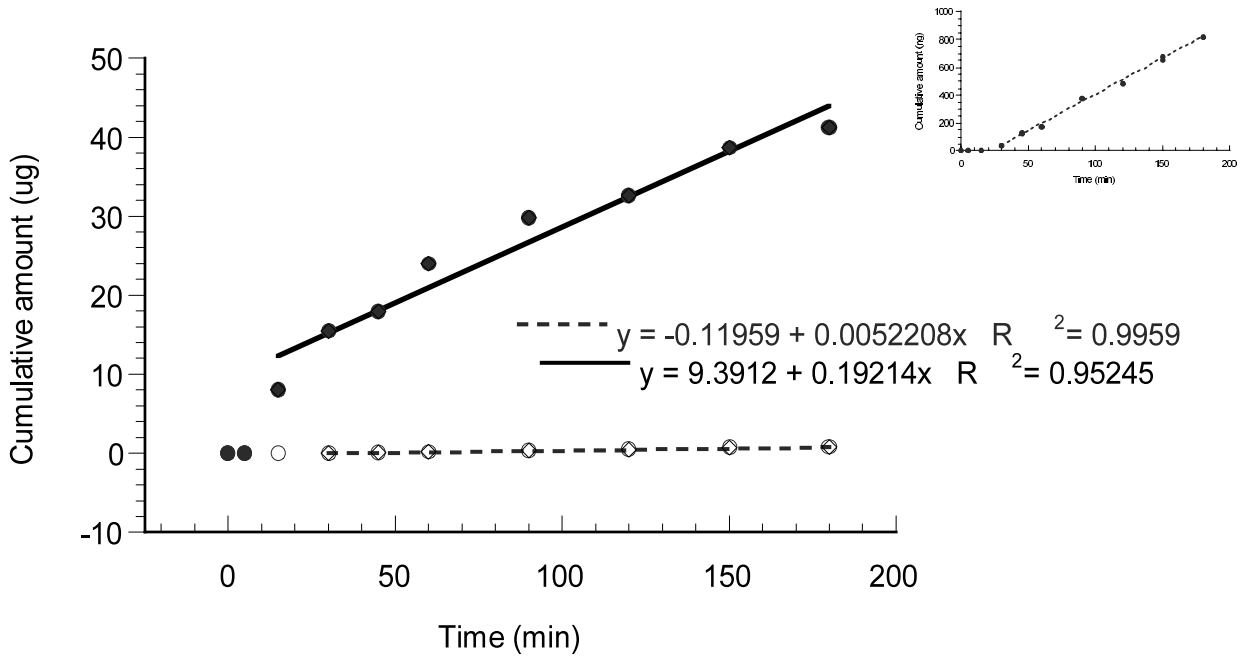

Fig. 5. Comparative in vitro transport profiles of diglycine (closed circle) and diaspartic acid (open diamond) conjugates of cosalane across SD rat small intestinal segment; ($n = 3$). Absorption enhancer: 20 mM desoxycholate.

ponential disposition with long plasma elimination half-lives (Fig. 10 and Table 3). The volume of distribution, and clearance values of both GC and ASPC are higher than those of cosalane (Table 3).

Mean plasma concentration profile after oral dosing of GC in 20 mM desoxycholate is shown in Fig. 11. After oral dosing, GC absorption appeared to be slow with no apparent lag time and the systemic concentration peaked at 260 min, at 2.54 μg/ml (Table 4). The absolute oral bioavailability was $5.10 \pm 1.51\%$.

4. Discussion

The amino acids and short peptides produced as a result of digestion of dietary proteins are absorbed by active transport mechanisms (Ganapathy et al., 1994). Drugs that are peptide derived, or are structurally related to peptides, are known to utilize the peptide transport system for GI absorption (Yang et al., 1999). Several drugs that are amino acids analogs exhibit good absorption characteristics, e.g. gabapentin (Stewart et al., 1993), baclofen (Cercos-Fortea et al., 1995) and

Table 1
Effect of various concentrations of sodium desoxycholate on the in vitro transport characteristics of ^{14}C-mannitol and ^{14}C-diazepam

Marker	P_{app} (10^{-5}) (cm/s)/(% transport)			
	Control (KBR)	5 mM bile salt	10 mM bile salt	20 mM bile salt
^{14}C-Mannitol	0.702 ± 1.155 (3.33)	2.428 ± 0.329 (7.07)	1.84 ± 0.045 (5.7)	15.5 (7)
^{14}C-Diazepam	0.190 (0.55)	ND	ND	2.417 ± 1.414 (5.7)

ND, not determined.

Table 2
In vitro transport characteristics of cosalane and its amino acid conjugates

Compound	P_{app} (cm/s)	
	Control	20 mM Desoxycholate
Cosalane	4.494×10^{-8}	$(7.159 \pm 0.273) \times 10^{-7}$
Di-aspartic acid conjugate	ND	$(7.756 \pm 0.003) \times 10^{-7}$
Di-glycine conjugate	ND	$(5.047 \pm 0.058) \times 10^{-5}$

ND, not detectable.

D-cycloserine (Thwaites et al., 1995). These compounds are transported by either a large neutral amino acid transporter (Cercos-Fortea et al., 1995) or by a proton coupled amino acid transporter (Thwaites et al., 1995). A number of amino acid transporters have been identified with different substrate specificities, depending on whether the amino acids are dipolar, or have acidic or basic side chains (Ganapathy et al., 1994). The diaspartic acid conjugate of cosalane may target the X_{AG}^{-} amino acid transport system. These conjugates retain good in vitro *anti*-HIV activities and, as a result, need not function as prodrugs of cosalane (Santhosh et al., 2000). Therefore, we investigated the absorption characteristics of both compounds using in vitro, in situ, and in vivo techniques in SD rat (excised intestinal segments and whole animal).

A variety of in vitro approaches have been employed to assess the transport properties of drug molecules across the intestinal epithelium. One of the most common methods uses isolated segments of the intestinal tissue to determine the intestinal permeability of a compound which is affected not only by the physiological properties of the mucosal membrane, but also physicochemical properties of the permeant. Therefore, permeability measurements are a useful tool in the prediction of drug absorption (Zheng et al., 1999).

Membrane permeation studies can be conducted in diffusion cells with excised tissue or cultured cells or by employing everted intestine technique or in situ methods like the closed loop (Aungst, 1993). The in vitro and in situ methods afford better control and the physiological and biopharmaceutical variables influencing drug bioavailability can be studied. The in situ closed loop method has been used to evaluate the effect of various formulations or formulation additives on intestinal absorption (Nakanishi et al., 1989). However, in case of a membrane interacting, poorly permeable compound like cosalane, in situ measurements of its luminal disappearance may not correlate with drug appearance in the blood. Therefore, both portal and jugular blood sampling was included in the experimental design to allow for a more complete pharmacokinetic analysis (Yorgey et al., 1986) and also to assess in vivo sequestration of the compound in the liver.

In order to delineate the involvement of amino acid transporters on the permeation of both GC and ASPC, in vitro transport studies employing segments from duodenum, jejunum, and ileum (proximal and distal regions) were performed. However, no regional differences in transport were seen and both conjugates failed to permeate the intestinal segments. In addition, the hydrolysis product-cosalane was also not found to any measurable extent. Therefore, both GC and ASPC failed to utilize the amino acid transporters and enhance the permeability of cosalane across the intestinal mucosa. These findings were further confirmed by the in situ closed loop method where the drug absorption was assessed from different regions of the small intestine.

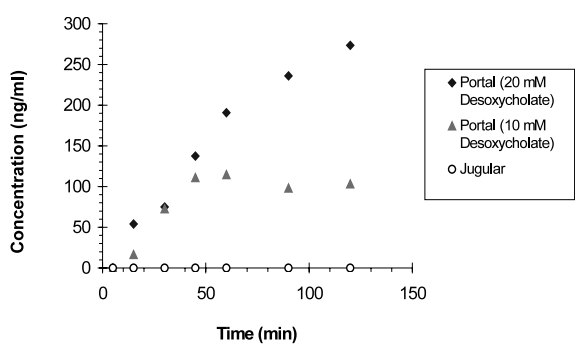

Fig. 6. In situ absorption profile of cosalane in the presence of 10 and 20 mM sodium desoxycholate formulations; ($n = 3$).

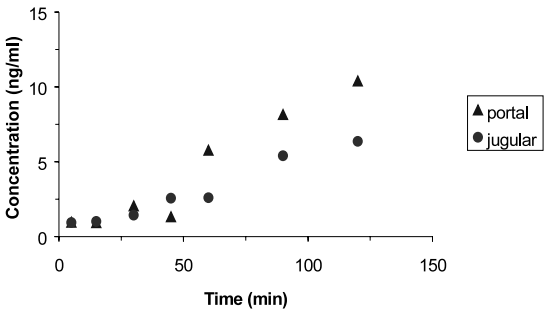

Fig. 7. In situ absorption profile of diglycine conjugate of cosalane in SD rat. Enhancer: 20 mM sodium desoxycholate. Dose 1.0 mg per rat.

Previous studies from our laboratory have shown that the transcellular permeability (P_{app}) of cosalane across Caco-2 cell monolayers was very low (4.5×10^{-8} cm/s). The diffusion of cosalane across the cells was extremely limited and is kinetically regulated by the equilibrium between the protein bound and free drug partitioning into the cell membrane. These studies suggest that cosalane, being membrane interactive, partitions and sequesters in the lipid bilayer of the cell membrane, which limits its permeation through the enterocytes (Pal et al., 2000). Another study from our laboratory reported on the metabolic stability of cosalane (Udata et al., 1999). In addition, preliminary studies to assess the stability of GC and ASPC in rat intestinal mucosal homogenates showed that neither of these two conjugates were enzymatically hydrolyzed to cosalane to any measurable extent.

Cosalane is extremely hydrophobic with a log P of 6.8. It has been generally accepted that increasing the hydrophobicity of a drug increases its permeability across the cell membrane (Lindahl et al., 1996). However, in an interesting study, Wils et al. have demonstrated that the permeability of a drug decreases if the log D (octanol/buffer distribution coefficient) values were greater than 3.5, indicating that increased hydrophobicity does not necessarily increase the ability of the molecule to be absorbed (Wils et al., 1994).

In an effort to improve the absorption of cosalane and its amino acid conjugates, sodium desoxycholate, an unconjugated bile salt, was chosen as a model absorption enhancer. The effect of various absorption enhancers on the transport characteristics of cosalane across Caco-2 cell monolayers was studied in our laboratory and desoxycholate was found to be an effective enhancer of cosalane transport, causing a minimal degree of mucosal cell disruption at concentrations below 20 mM (below the normal 40 mM bile salt concentration in human intestine at fed state). Based on these studies, various concentrations of desoxycholate (5–20 mM) were evaluated on the

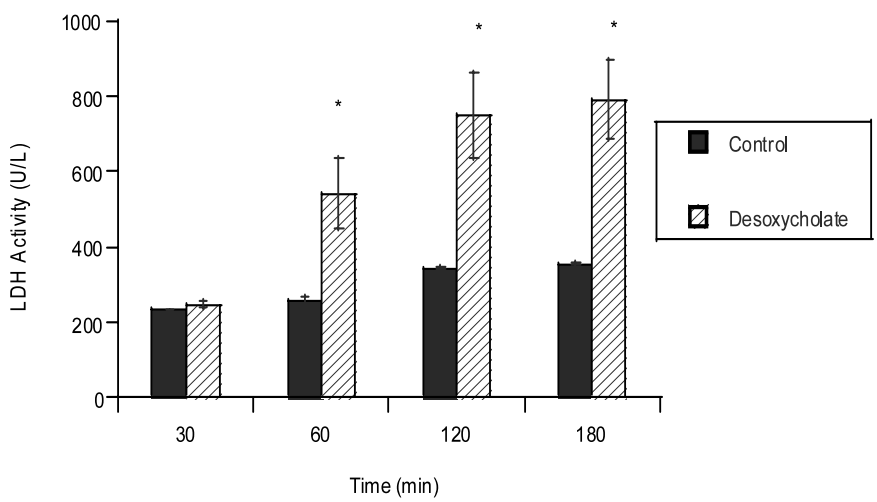

Fig. 8. Toxic effects of sodium desoxycholate in SD rat during in situ studies; ($n = 3$). *Statistically significant difference from the corresponding control values at $P < 0.05$.

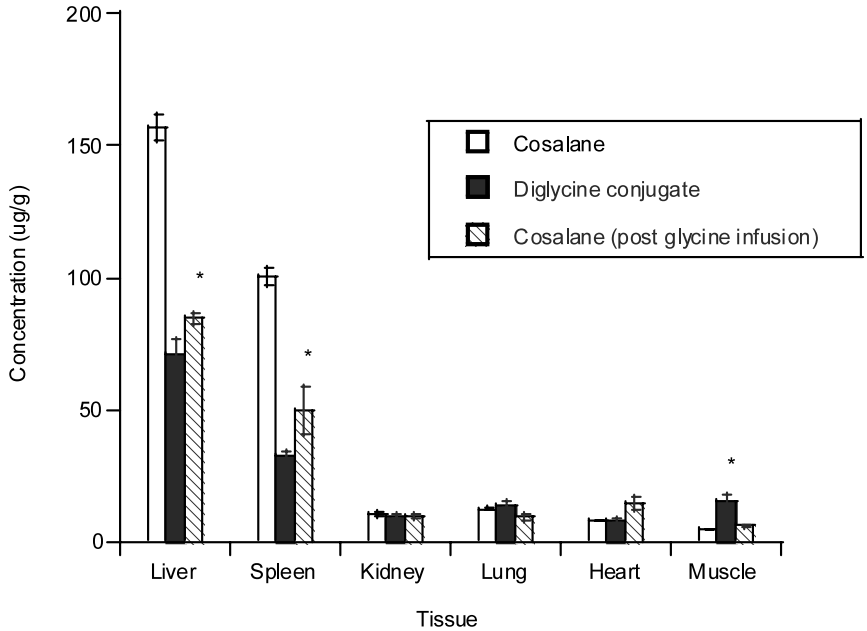

Fig. 9. Comparative tissue distribution of cosalane and diglycine conjugate in SD rat 2 h postdose; ($n = 3$). *Statistically significant difference from the respective cosalane tissue concentrations (control) at $P < 0.05$.

transport characteristics of cosalane, GC and ASPC across isolated intestinal segments. In addition the integrity and viability of the intestinal segment was assessed using [^{14}C]-mannitol and [^{14}C]-diazepam (Cogburn et al., 1991). Measurement of radiolabeled mannitol permeability is more sensitive to alterations in membrane integrity as small changes in permeability can be readily detected compared with the measurement of transepithelial electrical resistance (Milton and Knutson, 1990). Mannitol is a small diffusible marker and is routinely used as a paracellular permeability marker. Increased mannitol permeation may therefore indicate a loss in tight junctional integrity, which may also be due to a decrease in cell viability (Nejdfors et al., 2000). Diazepam is a small organic molecule that exhibits relatively high transport by the transcellular route (Cogburn et al., 1991). Both these markers are transported by passive diffusion across rabbit intestinal membrane (ileum) (Lee et al., 1997). The transport of [^{14}C]-mannitol increased 3–22 fold in the presence of 5, 10 and 20 mM desoxycholate indicating disruption of the tight junctions (Table 1). Moreover, the transport of [^{14}C]-diazepam increased >10 fold indicating the loss of integrity of the transcellular pathway (Table 1). Thus, the bile salt affects both the transcellular and paracellular routes. The effects of absorption enhancers, such as bile salts, on the transfer of solutes across the intestinal mucosa have been studied extensively. The bile salts increase solute absorption by altering the barrier function of the

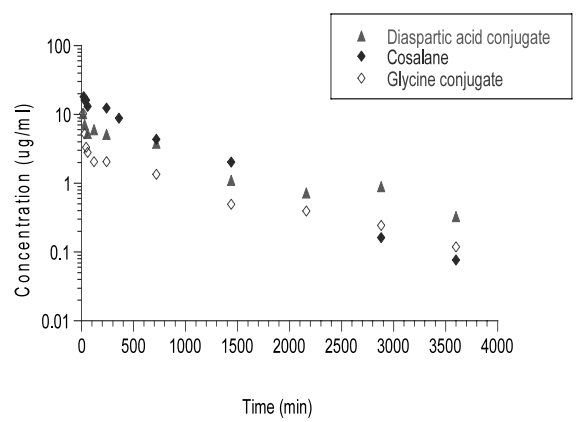

Fig. 10. Comparative intravenous profiles of cosalane and its amino acid conjugates in SD rat. Dose 1 mg/kg either in 10% ethanol or 5% DMSO; ($n = 3$).

Table 3
Pertinent intravenous pharmacokinetic parameters of cosalane and its amino acid conjugates in SD rat

Pharmacokinetic parameters	Cosalane (1 mg/kg)[a]	Diglycine conjugate (1 mg/kg)[b]	Diaspartic acid conjugate (1 mg/kg)[c]
AUC (μg min/ml)	11224 ± 1669*	3021 ± 276	5312 ± 118
Alpha half-life (min)	20 ± 17	11 ± 2.50	23 ± 3
Beta half-life (min)	555 ± 106	758 ± 99	815 ± 73
Clearance (ml/min)	0.0204 ± 0.002**	0.0809 ± 0.007**	0.0471 ± 0.002**
Volume of distribution (steady state) (ml)	15.40 ± 2.52***	78 ± 3.66***	54.10 ± 4.41***

* Statistically significant difference from b and c at $P<0.05$.
** Statistically significant difference from each other at $P<0.05$.
*** Statistically significant difference from each other at $P<0.05$.

cell membrane (Kakemi et al., 1970; Feldman et al., 1973); the mucus layer (Poelma et al., 1990); and the paracellular route (Yamashita et al., 1990; Lane et al., 1996; Werner et al., 1996). Therefore, the mechanism of absorption enhancement by bile salts appears to be complex. Increase in the cell membrane permeability by the bile salts appears to be associated with disorder in the interior of the membrane due to reverse micellization causing membrane destabilization.

The critical micellar concentration (CMC) of sodium desoxycholate has been reported to be 5 mM (Small, 1971). The transport of both mannitol and diazepam increases significantly at and above the CMC of desoxycholate, indicating some degree of lipid bilayer disruption. Similar results were obtained while investigating the effects of sodium deoxycholate on the transport of hydrophilic and hydrophobic model compounds in Caco-2 cell monolayers (Sakai et al., 1997). For enhanced drug absorption to occur, the enhancer must partition into the membrane and establish a concentration gradient that would be sufficient to disorder and solubilize more than 15–20% of the membrane lipids (LeCluyse et al., 1991). However, if the increased transport of GC were solely attributed to the membrane disruption, then the permeability values of both cosalane and ASPC should also be similar to that of GC. There are reports in the literature which indicate that the bile salts may decrease solute absorption by decreasing the thermodynamic activity of the drug as a result of its incorporation into micelles, causing a reduction in the free fraction of the drug due to solubilization (Poelma et al., 1989, 1990; Charman et al., 1997). Therefore, an increase in drug transport not only depends on the type of enhancer used but also on the physicochemical properties of the drug under study and the interaction of the bile salt with the membrane lipid.

Toxicity of desoxycholate (20 mM) was assessed during the in situ studies by monitoring the release of intracellular lactate dehydrogenase (LDH) into the surrounding milieu. The serum LDH activity increased more than two fold during the length of the experiment, suggesting some degree of tissue damage (Fig. 8).

In situ studies on the glycine conjugate show a lag time of 30 min (Fig. 7), which is consistent with the in vitro results (Fig. 5). Only 34% of the absorbed dose of the glycine conjugate was subjected to uptake by the reticuloendothelial (the spleen, liver and bone marrow) RES system. This

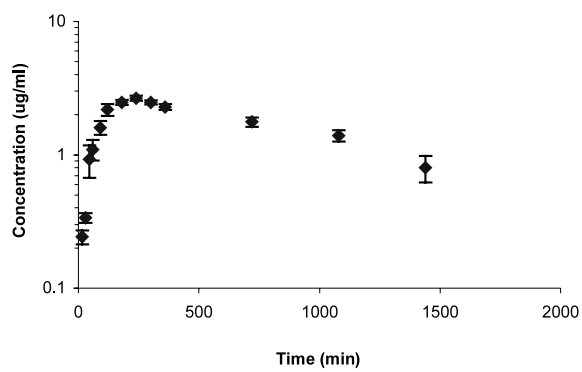

Fig. 11. Oral absorption profile of diglycine conjugate in SD rat. Absorption enhancer: 20 mm sodium desoxycholate; ($n=3$). Dose 20 mg/kg.

Table 4
Pharmacokinetic parameters of diglycine conjugate obtained after oral dosing to SD rat; ($n = 3$)

Parameter	Estimate ± SEM
AUC (μg min/ml)	3334 ± 280
$t_{\frac{1}{2}}$ (min)	718.33 ± 66.14
Cl/F (ml/min)	1.52 ± 0.12
t_{max} (min)	258.67 ± 7.54
C_{max} (mg/ml)	2.54 ± 0.05

is a significant finding since 100% of absorbed cosalane dose was sequestered by the RES and systemic levels were not observed. These findings corroborate earlier reports from our laboratory (Udata et al., 1999).

In light of improved GC systemic availability, tissue distribution (TD) studies of GC were conducted in the SD rat model. The animals were dosed at 10 mg/kg of GC in 20% DMSO and various tissues viz. liver, spleen, kidney, lung, heart and muscle were isolated, extracted and analyzed for GC. The extent of GC uptake by liver and spleen was significantly different ($P < 0.05$), and the concentrations of GC were $> 50\%$ lower compared to the concentrations of cosalane. Since, cosalane and GC differ only in the amino acid functionality, another study was conducted in which the rats were infused with the unnatural amino acid–glycine (300 mM; in 1.5 ml isotonic phosphate buffer, 1.5 h), followed by a bolus iv dose of cosalane (10 mg/kg). The tissues were isolated and analyzed for cosalane (Fig. 9). Cosalane distribution in the both the liver and spleen reduced to almost half of the control levels, suggesting that the glycine may play an important role in reducing the uptake of cosalane by the RES. These findings suggest that both cosalane and its glycine conjugate share common binding region(s) in the RE system. It is well known that Kupffer cells i.e. the macrophages in the liver play a principal role in the removal of gut derived endotoxin (lipopolysaccharide, LPS) (Nolan, 1981). Endotoxins like LPS activate the Kupffer cells and aid in their macrophagic activity. Glycine has been shown to protect the proximal tubules and hepatocytes against hypoxia (Lemasters and Thurman, 1997). Schemmer et al. have demonstrated that glycine prevents the activation of Kupffer cells (Schemmer et al., 1999). They proposed that protective effect of glycine is most likely related to the actions on the glycine–gated chloride channels in Kupffer cells. According to the working hypothesis of Schemmer et al., glycine activates a glycine-gated chloride channel in the membrane of the Kupffer cells leading to an influx of chloride ions that cause subsequent hyperpolarization of the membrane which prevents the activation of the Kupffer cells. However, this hypothesis is still under investigation.

In order to build on these findings, oral studies of GC were performed in 20 mM desoxycholate formulation. Fig. 11 illustrates the oral profile of GC. Although, the oral profile shows no apparent lag time, the absorption of GC is a slow process, and the C_{max} is reached at around 258.6 ± 7.6 min. The elimination is slow and the elimination $t_{1/2}$ obtained from both the iv study (758 ± 99 min), and oral study (718 ± 66) are in good agreement with each other. With an absolute oral bioavailability of 5.10 ± 1.51%, the glycine conjugate appears to be promising lead compound. GC appears to be distributed to some extent into the peripheral tissues that are not highly perfused, e.g. muscle (Fig. 11). Thus, a higher value of V_d for GC in comparison to cosalane is not surprising (Table 3). Although the clearance of GC is higher than that of cosalane, further studies (metabolic and elimination) are needed to better understand the pharmacokinetics of GC.

Based on these in vitro, in situ and in vivo studies, the glycine conjugate appears to be a promising *anti*-AIDS lead compound for further development as it has the capability to escape uptake by the RES. In addition it has good in vitro *anti*-HIV activity and is not required to function as a prodrug.

4.1. Future recommendations

A long term objective of this project is to investigate strategies to improve the absolute oral bioavailability of cosalane. Based on the finding of these studies the following recommendations are proposed to improve cosalane's oral bioavailability.

4.1.1. Replacement of the cholestane moiety of cosalane with a bile acid moiety

The utilization of carrier-mediated transport mechanisms may serve as a useful and productive strategy to enhance intestinal absorption. Bile acid transporters may be exploited to achieve this goal. Bile acid transporters are known to possess favorable characteristics such as low substrate specificity and high capacity. It has been demonstrated that the high capacity of bile acid transporters cause rapid uptake of 3-tosylcholic acid in rat ileum which is similar to cholic acid and 3-benzoylcholic acid (Ho, 1987). In addition to high capacity, the bile acid transporters have been shown to accept and transport a variety of analogs that are derived at the 3- and 24-positions (Ho, 1987; Swaan et al., 1997). The rate of transport in the ileum depends on the number of hydroxyl groups, with trihydroxy > dihydroxy > monohydroxy (Lack and Weiner, 1966; Schiff et al., 1972; Aldini et al., 1992; Swaan et al., 1997). No single hydroxyl group appears to be critical for recognition by the bile acid transport system and that among the dihydroxy compounds, there is little difference in the active transport rates (Ho, 1987). Therefore, the bile acid transport system can be utilized to facilitate the transport of poorly membrane permeable compounds like cosalane and its analogs. The present hypothesis regarding the inability of cosalane to penetrate biological membranes suggests that the lipophilic steroid moiety, cholestane, embeds in the membrane, causing the molecule to be sequestered in the lipid bilayer. By modifying or changing the cholestane to a bile acid moiety, the compound can be rendered more polar thereby allowing the resultant molecule to pass through the membrane via the bile acid transporter.

4.1.2. Utilization of a combination approach

It is possible to conjugate amino acids and short peptides/peptidomimetics to the pharmacophore (carboxyl groups) and modify/replace the cholestane moiety with bile acids. This approach would exploit both the amino acid and bile acid transporters and most likely enhance the oral bioavailability of the molecule. The glycine conjugate of cosalane appears to be a very promising candidate for this approach. The rate-limiting step in achieving high in vivo systemic bioavailability appears to be its poor membrane permeation. Therefore, replacement of the cholestane moiety of GC with a bile acid may overcome its poor intestinal permeability problem.

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References

Aldini, R., Roda, A., Lenzi, P.L., Ussia, G., Vaccari, M.C., Festi, D., Bazzoli, F., Galletti, G., Casanova, S., 1992. Bile acid active and passive ileal transport in the rabbit: effect of luminal stirring. Eur. J. Clin. Invest. 22 (11), 744–750.

Aungst, B.J., 1993. Novel formulation strategies for improving oral bioavailability of drugs with poor membrane permeation or presystemic metabolism. J. Pharm. Sci. 82, 979–987.

Cercos-Fortea, T., Polache, A., Nacher, A., Cejudo-Ferragud, E., Casabo, V.G., Merino, M., 1995. Influence of leucine on intestinal baclofen absorption as a model compound of neutral alpha-aminoacids. Biopharm. Drug Dispos. 16, 563–577.

Charman, W.N., Porter, C.J., Mithani, S., Dressman, J.B., 1997. Physiochemical and physiological mechanisms for the effects of food on drug adsorption: the role of lipids and pH. J. Pharm. Sci. 86 (3), 269–282.

Cogburn, J.N., Donovan, M.G., Schasteen, C.S., 1991. A model of human small intestinal absorptive cells. 1. Transport barrier. Pharm. Res. 8, 210–216.

Cushman, M., Golebiewski, W.M., McMahon, J.B., Buckheit, R.W.J., Clanton, D.J., Weislow, O., Haugwitz, R.D., Bader, J., Graham, L., Rice, W.G., 1994. Design, synthesis and biological evaluation of cosalane a novel anti-HIV agent which inhibits multiple features of virus replication. J. Med. Chem. 37, 3040–3050.

Feldman, S., Reinhard, M., Willson, C., 1973. Effect of sodium taurodeoxycholate on biological membranes: release of phosphorus, phospholipid, and protein from everted rat small intestine. J. Pharm. Sci. 62, 1961–1964.

Ganapathy, V., Brandsch, M., Leibach, F.H., 1994. Intestinal Transport of Amino Acids and Peptides. Raven Press, New York.

Ho, N.F., 1987. Utilizing bile acid carrier mechanisms to enhance liver and small intestine absorption. Ann. NY Acad. Sci. 507, 315–329.

Kakemi, K., Sezaki, H., Konishi, R., Kimura, T., Okita, A., 1970. Effect of bile salts on the gastrointestinal absorption of drugs. II. Mechanism of the enhancement of the intestinal absorption of sulfaguanidine by bile salts. Chem. Pharm. Bull. 18, 1034–1039.

Kuchimanchi, K.R., Udata, C., Johnston, T.P., Mitra, A.K., 2000. Pharmacokinetics, biliary excretion, and tissue distribution of novel anti-HIV agents, cosalane and dihydrocosalane, in Sprague–Dawley rats. Drug Metab. Dispos. 28, 403–408.

Lack, L., Weiner, I.M., 1966. Intestinal bile salt transport: structure-activity relationships and other properties. Am. J. Physiol. 210 (5), 1142–1152.

Lane, M.E., O'Driscoll, C.M., Corrigan, O.I., 1996. The relationship between rat intestinal permeability and hydrophilic probe size. Pharm. Res. 13, 1554–1558.

LeCluyse, E.L., Appel, L.E., Sutton, S.C., 1991. Relationship between drug absorption enhancing activity and membrane perturbing effects of acylcarnitines. Pharm. Res. 8, 84–87.

Lee, C.P., de Vrueh, R.L.A., Smith, P.L., 1997. Selection of development candidates based on in vitro permeability measurements. Adv. Drug Delivery Rev. 23, 47–62.

Lemasters, J.J., Thurman, R.G., 1997. Reperfusion injury after liver preservation for transplantation. Annu. Rev. Pharmacol. Toxicol. 37, 327–338.

Lindahl, A., Sandstrom, R., Ungell, A.L., Abrahamsson, B., Knutson, T.W., Knutson, L., Lennernas, H., 1996. Jejunal permeability and hepatic extraction of fluvastatin in humans. Clin. Pharmacol. Ther. 60, 493–503.

Milton, S.G., Knutson, V.P., 1990. Comparison of the function of the tight junctions of endothelial cells and epithelial cells in regulating the movement of electrolytes and macromolecules across the cell monolayer. J. Cell. Physiol. 144, 498–504.

Nakanishi, K., Masada, M., Nadai, T., Miyajima, K., 1989. Effect of the interaction of drug–beta-cyclodextrin complex with bile salts on the drug absorption from rat small intestinal lumen. Chem. Pharm. Bull. 37, 211–214.

Nejdfors, P., Ekelund, M., Jeppsson, B., Westrom, B.R., 2000. Mucosal in vitro permeability in the intestinal tract of the pig, the rat, and man: species- and region-related differences. Scand. J. Gastroenterol. 35, 501–507.

Nolan, J.P., 1981. Endotoxin, reticuloendothelial function, and liver injury. Hepatology 1, 458–465.

Pal, D., Udata, C., Mitra, A.K., 2000. Transport of cosalane—a highly lipophilic novel anti-HIV agent—across caco-2 cell monolayers. J. Pharm. Sci. 89, 826–833.

Poelma, F.G., Breas, R., Tukker, J.J., 1990. Intestinal absorption of drugs. III. The influence of taurocholate on the disappearance kinetics of hydrophilic and lipophilic drugs from the small intestine of the rat. Pharm. Res. 7, 392–397.

Poelma, F.G., Tukker, J.J., Crommelin, D.J., 1989. Intestinal absorption of drugs. I: The influence of taurocholate on the absorption of dantrolene in the small intestine of the rat. J. Pharm. Sci. 78, 285–289.

Sakai, M., Imai, T., Ohtake, H., Azuma, H., Otagiri, M., 1997. Effects of absorption enhancers on the transport of model compounds in Caco-2 cell monolayers: assessment by confocal laser scanning microscopy. J. Pharm. Sci. 86, 779–785.

Santhosh, K.C., Paul, G.C., De Clercq, E., Pannecouque, C., Witvrouw, M., Loftus, T.L., Turpin, J.A., Buckheit, R.W.J., Cushman, M., 2001s. Correlation of anti-HIV activity with anion spacing in a series of cosalane analogs with extended polycarboxylate pharmacophores. J. Med. Chem. 44 (5), 703–714.

Santhosh, K.C., De Clercq, E., Pannecouque, C., Witvrouw, M., Loftus, T.L., Turpin, J.A., Buckheit, R.W. Jr., Cushman, M., 2000. Anti-HIV activity of a series of cosalane amino acid conjugates. Bioorg. Med. Chem. Lett. 10, 2505–2508.

Schemmer, P., Bradford, B.U., Rose, M.L., Bunzendahl, H., Raleigh, J.A., Lemasters, J.J., Thurman, R.G., 1999. Intravenous glycine improves survival in rat liver transplantation. Am. J. Physiol. 276, G924–G932.

Schiff, E.R., Small, N.C., Dietschy, J.M., 1972. Characterization of the kinetics of the passive and active transport mechanisms for bile acid absorption in the small intestine and colon of the rat. J. Clin. Invest. 51 (6), 1351–1362.

Small, D.M., 1971. The physical chemistry of cholanic acids. In: Nair, P.P., Kritchevsky, D. (Eds.), The Bile Acids, Chemistry, Physiology, and Metabolism. Plenum Press, New York, pp. 249–356.

Stewart, B.H., Kugler, A.R., Thompson, P.R., Bockbrader, H.N., 1993. A saturable transport mechanism in the intestinal absorption of gabapentin is the underlying cause of the lack of proportionality between increasing dose and drug levels in plasma. Pharm. Res. 10, 276–281.

Swaan, P.W., Hillgren, K.M., Szoka, F.C. Jr., Oie, S., 1997. Enhanced transepithelial transport of peptides by conjunction to cholic acid. Bioconjug. Chem. 8 (4), 520–525.

Thwaites, D.T., Armstrong, G., Hirst, B.H., Simmons, N.L., 1995. D-cycloserine transport in human intestinal epithelial (Caco-2) cells mediated by a H^+-coupled amino acid transporter. Br. J. Pharmacol. 115, 761–766.

Udata, C., Mitra, A.K., Badr, M.Z., 1999. Disposition of cosalane, a novel anti-HIV agent, in isolated perfused rat livers. Drug Metab. Dispos. 27, 947–950.

Werner, U., Kissel, T., Reers, M., 1996. Effects of permeation enhancers on the transport of a peptidomimetic thrombin inhibitor (CRC 220) in a human intestinal cell line (Caco-2). Pharm. Res. 13, 1219–1227.

Wils, P., Warnery, A., Phung-Ba, V., Legrain, S., Scherman, D., 1994. High lipophilicity decreases drug transport across intestinal epithelial cells. J. Pharmacol. Exp. Ther. 269, 654–658.

Yamashita, S., Masada, M., Nadai, T., Kimura, T., 1990. Effect of adjuvants on charge-selective permeability and electrical resistance of rat jejunal membrane. J. Pharm. Sci. 79, 579–583.

Yang, C.Y., Dantzig, A.H., Pidgeon, C., 1999. Intestinal peptide transport systems and oral drug availability. Pharm. Res. 16, 1331–1343.

Yorgey, K.A., Pritchard, J.F., Renzi, N.L., Dvorchik, B.H., 1986. Evaluation of drug absorption and presystemic metabolism using an in situ intestinal preparation. J. Pharm. Sci. 75, 869–872.

Zheng, Y., Qiu, Y., Lu, M.F., Hoffman, D., Reiland, T.L., 1999. Permeability and absorption of leuprolide from various intestinal regions in rabbits and rats. Int. J. Pharm. 185, 83–92.