Intestinal Transport of Gentamicin with a Novel, Glycosteroid Drug Transport Agent

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Purpose. The objective was to investigate the ability of a glycosteroid (TC002) to increase the oral bioavailability of gentamicin.
Methods. Admixtures of gentamicin and TC002 were administered to the rat ileum by injection and to dogs by ileal or jejunal externalized ports, or PO. Bioavailability of gentamicin was determined by HPLC. ^{3}H-TC002 was injected via externalized cannulas into rat ileum or jejunum, or PO and its distribution and elimination was determined. The metabolism of TC002 in rats was evaluated by solid phase extraction and HPLC analysis of plasma, urine and feces following oral or intestinal administration.
Results. The bioavailability of gentamicin was substantially increased in the presence of TC002 in both rats and dogs. The level of absorption was dependent on the concentration of TC002 and site of administration. Greatest absorption occurred following ileal or jejunal administration. TC002 was significantly more efficacious than sodium taurocholate, but similar in cytotoxicity. TC002 remained primarily in the GI tract following oral or intestinal administration and cleared rapidly from the body. It was only partly metabolized in the GI tract, but was rapidly and completely converted to its metabolite in plasma and urine.
Conclusions. TC002 shows promise as a new drug transport agent for promoting intestinal absorption of polar molecules such as gentamicin.

KEY WORDS: gentamicin; absorption enhancer; oral drug delivery; bile salts.

INTRODUCTION

Gentamicin is an important aminoglycoside antibiotic for treatment of infections caused by a wide spectrum of aerobic gram-negative bacilli and gram-positive cocci (1,2). However, oral delivery has not been possible because less than 1% of orally administered gentamicin is systemically absorbed (3).

Bile acids are a class of widely investigated agents that facilitate the movement of polar molecules through biological membranes (4–7). Carey and coworkers (4) proposed that bile acids form small aggregates through the interaction of their polar surfaces. They further suggested that these aggregates form an ordered hydrophilic channel or water-filled defects in the membrane through which polar molecules could pass.

The importance of bile acid polarity in membrane transport was examined by constructing analogs of cholic acid with sugars in the C-7 and C-12 positions of the steroid nucleus, thereby increasing overall hydrophilicity as well as polarity at the hydrophilic surface (8). One of the analogs (TC002, Figure 1) was found to effectively promote the intestinal absorption of gentamicin, calcitonin and vancomycin and nasal absorption of insulin. Furthermore, TC002 caused far less damage to erythrocyte membranes than deoxycholic and taurocholic acids. The interesting properties of TC002 prompted us to conduct additional investigations.

MATERIALS AND METHODS

Materials

All chemicals were from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated. TC002 (MW 746) was synthesized and purified to greater than 90% at Intercardia (8,9). ^{3}H-TC002, of 70% radiochemical purity, was prepared at SRI International from ^{3}H-cholic acid (^{3}H at C-2 and C-4) using the same synthetic protocol. All solvents were analytical grade and purchased from Curtin Matheson Scientific, Inc. (Houston, TX).

Gentamicin Absorption

Fasted, Sprague-Dawley rats (250–300 g) were anesthetized throughout the study with 60 mg/kg ketamine and 8 mg/kg xylazine. Compounds dissolved in 0.5 ml water were injected into the terminal ileal segment, 5 cm above the cecum. Intravenous solutions, in 0.3 ml water, were administered through a jugular vein cannula.

Dogs (20–30 kg) were implanted with intestinal access ports (Access Technologies, Skokie, IL) on their backs, and tubing of 0.8 mm inner diameter led to either the jejunum (midway between the pylorus and the cecum) or the ileum (10–20 cm from the cecum). Fasted animals were dosed with water solutions: 5 ml through intestinal ports or 1 ml intravenously through the leg vein.

Blood samples of 0.6 ml in rats and 2.5 ml in dogs were withdrawn from the implanted jugular (rats) or femoral (dogs) vein cannulas and replaced with an equal volume of heparinized saline (10 units/ml). Plasma was separated and stored at −20°C. Gentamicin concentration in plasma was determined by HPLC (10).

All animal experiments adhered to the "Guide and Use of Laboratory Animals" and performed under a written protocol approved by the Univ. of Michigan's Laboratory of Animal Medicine.

TC002 Absorption, Elimination and Metabolism

Male Sprague Dawley rats (150–200 g) were implanted with jugular, ileal or jejunal cannulas. The ileal cannula was placed 25 cm proximal to the ileo-cecal junction and the jejunal cannula was positioned 3 cm distal to the point where the duodenum curves towards the midline. For absorption and elimination studies, 120.5 μCi^{3}H/kg was dissolved in a volume of 2. 5 ml/kg 0.9% sterile saline. Intravenous formulations consisted of 110 μCi ^{3}H/kg in 1 ml/kg saline. For metabolic studies, a 40 mg/ml saline solution was injected into the ileal cannula placed 10 cm proximal to the ileo-cecal junction.

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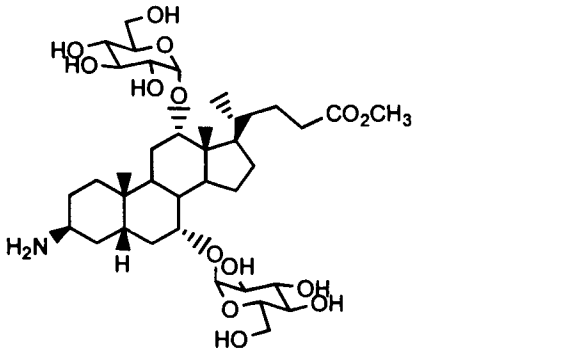
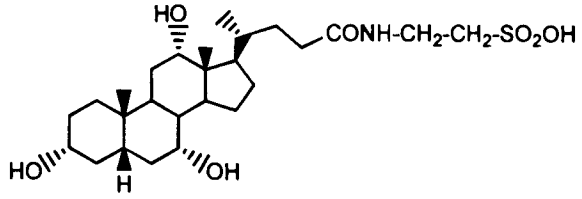


TC002



Taurocholic acid

Fig. 1. Structures of TC002 and taurocholic acid.

Approximately 200 μl of blood was collected from either the tail vein or femoral vein into heparinized tubes. Plasma was separated and stored at −20°C. Urine, cage rinse and feces were stored at −80°C. In the elimination studies, animals were euthenized at 72 hr and the liver, bile duct, stomach, small intestine, large intestine and the gastrointestinal (GI) tract contents were removed. Tissues, feces and GI contents were weighed, homogenized and combusted prior to scintillation counting. The carcass was dissolved in 5M KOH dissolved in 50% MeOH and samples were combusted prior to counting.

All animal studies were conducted in accordance with the NRC "Guide for the Care and Use of Laboratory Animals" and the USDA "Laboratory Animal Welfare Act". The study protocol was approved by IACUC.

Calculation of Intestinal Absorption

Absolute bioavailability (%) was calculated using the equation,

$$\% \text{ Bioavailability} = \frac{(\text{AUC}) \text{ ileal (Dose)iv}}{(\text{AUC})\text{iv (Dose)ileal}} \times 100$$

where AUC is the area under the plasma concentration-time curve from 0 to 240 minutes.

Statistical Analysis

Means of two groups were compared using the non-paired Student t-test. When comparing multiple groups, we used one way analysis of variance combined with the Tukey multiple comparison procedure.

Analysis of TC002 in Biological Fluids

Plasma, urine and 20% fecal samples were centrifuged (14,000 × g, 5 min), diluted with water (20X) and extracted with a C18 SPE cartridge (Extract Clean, 100 mg, Alltech). The sample was eluted using a 3 step gradient: water, 10% acetonitrile in 12.5 mM potassium phosphate (pH 7), ending with 60% acetonitrile in 12.5 mM potassium phosphate (pH 7). The final eluate was diluted 1:1 with water and ultrafiltered (5kd Ultrafree-MC, Millipore). The samples were reacted with OPA (OPA, fluoraldehyde reagent: Pierce, Rockford, IL) for 4.5 min prior to injection onto a C18 Supelcosil LC-18-DB column (4.6 mm × 25 cm, 5 μm, Supelco). The C18 column was pre-equilibrated with mobile phase A (30% acetonitrile, 7% tetrahydrofuran, 7.875 mM potassium phosphate pH 7.0). The sample was eluted for 5 minutes (at 2 ml/min) in mobile phase "A" followed by a 0%–80% 15 minute linear gradient of mobile phase "B" (acetonitrile). The elution buffer was held at 80% mobile phase "B" for an additional 5 minutes before re-equilibration of the column in mobile phase "A". OPA conjugates were detected using 330 nm excitation and 450 nm emission settings.

Cytotoxicity

Cytotoxicity was measured by a standard MTT assay (11) using LC-PK_1 pig kidney epithelial cells. Cells were exposed to TC002 or Na taurocholate in OptiMEM for 6 hours and then incubated for 24 hours in growth medium containing 0.2 mg/ml MTT. The cytotoxicity was determined by linear regression analysis and expressed as the concentration of compound that produced 50% cytotoxicity (IC_{50}).

Histopathology

A segment of the ileum below the injection point was obtained from rats treated as described. Tissue samples from 1–2 rats dosed with either buffer, 30, 50 or 100 mM TC002 or 100 mM Na taurocholate were immediately fixed in formalin, embedded in paraffin wax and sectioned. The sections were stained with hematoxalin and eosin and examined by light microscopy.

RESULTS

Absorption in Rats

A mixture of TC002 and gentamicin was injected into the ileum of anesthetized rats and the circulating levels of gentamicin were monitored. In the absence of TC002, a Cmax of 0.6 μg/ml is observed after 7 minutes and levels decline gradually over a period of 4 hours (Figure 2). In contrast, when the same dose of gentamicin is admixed with 100 mM TC002, peak plasma levels reach 7.3 μg/ml. Substantial plasma levels are observed within 5 minutes. Cmax is reached within 20 minutes and maintained until 90 minutes. Clearance is very slow and high levels of gentamicin (5.5 μg/ml) are still circulating at 4 hours. In contrast, *iv* administration leads to very rapid clearance: in 5 minutes peak blood levels were reduced in half.

When the ratio of TC002 to gentamicin is reduced, while retaining a fixed amount of gentamicin, absorption is decreased while the pharmacokinetic profile remains (Figure 2). The kinetics of gentamicin uptake with 10, 50 and 100 mM TC002 are similar, but the Cmax increases with the increased concentration of TC002. Bioavailabilities of 43.6, 72.3 and 109.7% were obtained when 10, 50 and 100 mM TC002 was used with 5

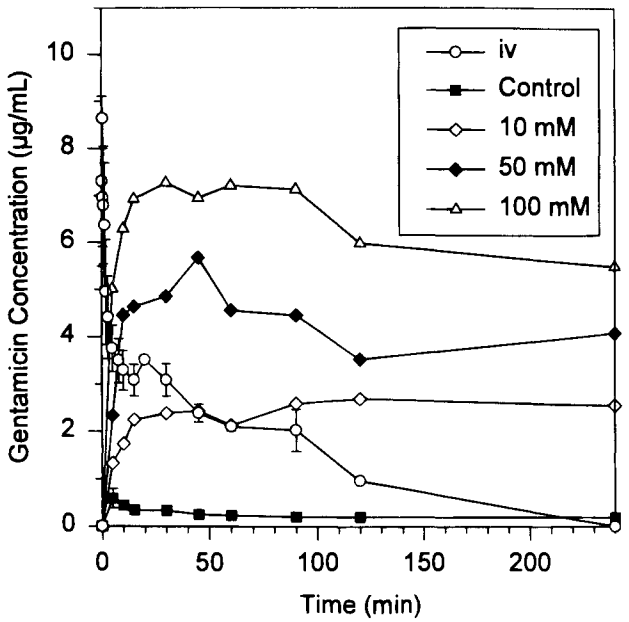


Fig. 2. Plasma gentamicin levels in rats following ileal administration of 5 mg (17–20 mg/kg) of gentamicin formulated in the presence of either 0 (Control), 10, 50 or 100 mM TC002 or *iv* administration of 1 mg (5mg/kg) gentamicin. Mean of 3–4 rats.

mg (17–20 mg/kg) of gentamicin (Table I). These values are 10 to 28 times higher than the 4.1% bioavailability of gentamicin administered in the absence of TC002. Although substantial uptake of gentamicin occurs even with a TC002: gentamicin molar ratio of 0.5:1 (10 mM TC002 and 5 mg gentamicin), a ratio of 5:1 is necessary to transport virtually all the drug.

It is well known that bile acids are capable of enhancing the uptake of drugs in the intestine (12–14). Since TC002 is a glycosylated derivative of cholic acid, we thought it would be informative to compare TC002 with taurocholate. Taurocholate and TC002 share the cholic acid steroid nucleus but taurocholate contains hydroxyls at the C-7 and C-12 positions instead of the more hydrophilic glucose units in TC002. TC002 also contains an amine at the C-3 position instead of a hydroxyl. The two also differ in the side chain in the C-24 position: TC002 contains a methyl ester while taurocholate contains a taurine unit (Figure 1). The two compounds exhibit a similar, low cytotoxicity with LC-PK1 pig kidney epithelial cells in culture: IC_{50} of 9.4 mM ± 2.7 SD for TC002 and 15.5 mM ± 0.25 SD for taurocholate. Histopathology of rat ileum 2–4 hours following ileal injection of TC002 or taurocholate suggests that both compounds produce similar, mild effects.

Table I. Percent Bioavailability of Gentamicin Administered via Rat Ileum[a]

Concentration of delivery agent (mM)	TC002	Sodium taurocholate
0	4.1 ± 1.4	4.1 ± 1.4
10	43.6 ± 16.4	24.1 ± 10.3
50	72.3 ± 7.0*	28.3 ± 15.4
100	109.7 ± 15.2**	30.4 ± 6.8

[a] Gentamicin was mixed with TC002 or taurocholate and a final gentamicin dose of 5 mg/0.5 ml (17–20 mg/kg) was administered to each animal. The dose of TC002 varied from 3.7 mg (10 mM) to 37.3 mg (100 mM) per rat. Values are Mean ± SEM of 3–4 animals. Statistically significant differences between the 0 and higher enhancer concentrations are indicated as * for P= 0.022 and ** for P=0.001.

The pharmacokinetics of gentamicin in the presence of taurocholate and TC002 are quite different (Figure 3). Gentamicin is absorbed with similar kinetics but the apparent clearance rate is significantly faster (P = 0.049) when taurocholate is used. The Cmax is lower (4.5 µg/ml) with 100 mM taurocholate and this difference is reflected in gentamicin bioavailability (Table I). Whereas gentamicin is completely absorbed in the presence of 100 mM TC002, only 30% is absorbed with the same concentration of taurocholate and this difference is statistically significant (P = 0.008).

Absorption in Dogs

The applicability of TC002 to gentamicin transport in a larger and non-anesthetized animal was examined by using dogs implanted with externalized intestinal ports. The TC002-gentamicin mixture was introduced through the ileum, jejunum or by oral gavage. The initial pharmacokinetics of ileal absorption are similar in dogs with 134 mM TC002 and in rats with 100 mM TC002. In dogs, maximal uptake occurs in the first 20 minutes and the Cmax is maintained for approximately 40 minutes (Figure 4). However, clearance occurs more rapidly in dogs than in rats. By 4 hours most of the drug has disappeared from the circulation and levels drop to almost baseline. Absorption of gentamicin (5 mg/kg) is considerably increased in the presence of TC002: Cmax rises from 0.5 µg/ml in the absence of TC002 to almost 5 µg/ml in the presence of 134 mM TC002. Bioavailability rises by nine-fold: from 6 to 53% (Table II).

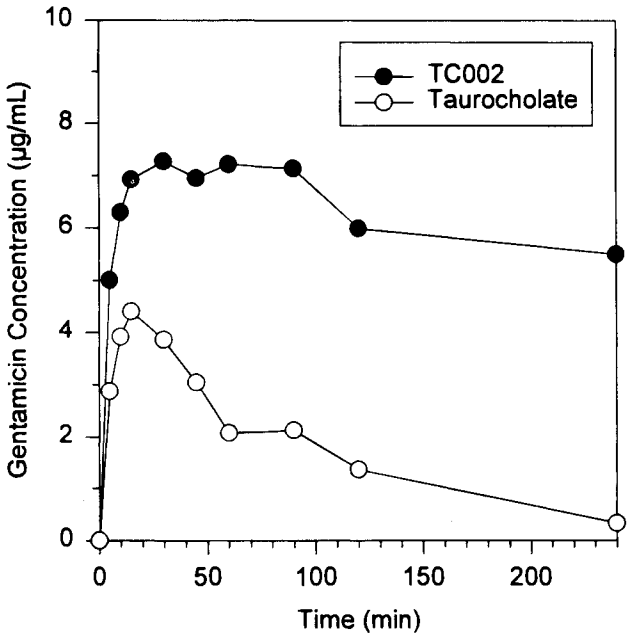


Fig. 3. Plasma gentamicin levels in rats following ileal administration of 5 mg (17–20 mg/kg) of gentamicin formulated with either 100 mM TC002 or 100 mM sodium taurocholate. Mean of 3–4 rats.

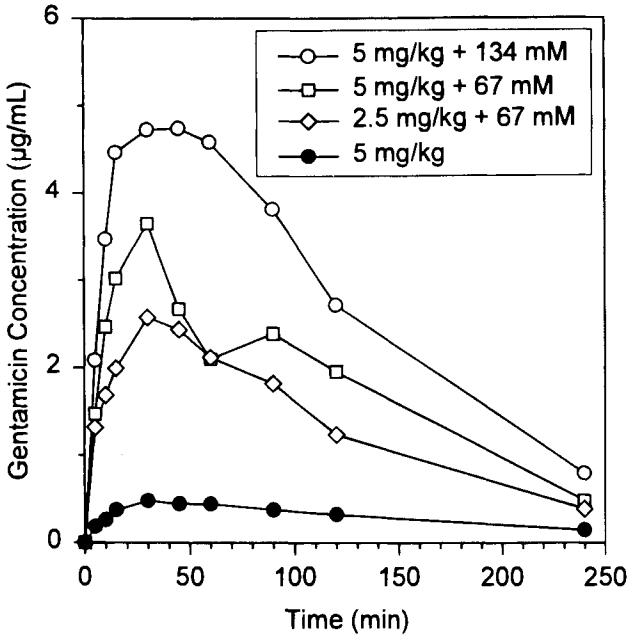


Fig. 4. Plasma gentamicin levels in dogs following ileal administration of gentamicin formulated with different concentrations of TC002. Gentamicin was either formulated at 5mg/kg (100 mg) without TC002 (●), 2.5 mg/kg (50 mg) with 67 mM TC002 (◇), 5 mg/kg (100 mg) with 67 mM TC002 (□), or 5mg/kg (100 mg) with 134 mM TC002 (O). Mean of 3–4 dogs.

TC002: gentamicin ratio and sites of administration were varied to gain a better understanding of TC002 action. With a molar ratio of 5:1 (134 mM TC002 and 5 mg/kg gentamicin) the absolute bioavailability was 53%. When 5:1 molar ratio was maintained but the TC002 dose reduced to 67 mM, the bioavailability was not significantly changed (61.8%). This observation suggests that the efficiency of absorption may be related to the ratio of the drug and TC002.

We compared the effect of introducing the drug mixture via the ileal or jejunal ports, or by oral gavage. Jejunal and oral routes proved to be less suitable than the ileum (Table II). In the jejunum, only 22.6% of gentamicin was absorbed. However, levels above 1 µg/ml were maintained for 2 hours. Oral administration resulted in bioavailability of only 9.4–10.6%, with no difference between 67 mM and 134 mM TC002 concentrations. However, while bioavailability was lower than in the ileum, TC002 increased the basal bioavailability by approximately 5 fold at both sites. The potency of TC002 was reduced but not eliminated in these more difficult sites.

Table II. Percent Bioavailability of Gentamicin Administered to Dogs[a]

Route of administration	TC002 concentration (mM)	Gentamicin dose (mg)	Bioavailability (%)
Ileum	0	100	6.1 ± 1.6
	67	100	31.5 ± 16.8
	134	100	53.5 ± 11.0*
	67	50	61.8 ± 3.5*
Jejunum	0	100	4.2 ± 0.3
	134	100	22.6 ± 5.2*
Oral	0	100	1.6 ± 0.3
	67	100	10.6 ± 1.5**
	134	100	9.4 ± 0.8**

[a] Gentamicin was mixed with TC002 and a 5 ml volume of each mixture was administered to each animal. The dose of TC002 was either 67 mM (250 mg) or 134 mM (500 mg). Values are Mean ± SEM of 3–4 animals. Statistically significant differences between the 0 and higher TC002 concentrations, for each route, are indicated as * for $P \leq 0.02$ and ** for $P \leq 0.008$.

Absorption, Pharmacokinetics and Elimination of Radiolabeled TC002

The distribution and elimination of TC002 was examined by studying the fate of ^{3}H-TC002. The dose and volume of TC002 in these studies (54 mM) was similar to the intermediate (50 mM) dose used in the gentamicin absorption studies. Small amounts of TC002 were very rapidly absorbed into the blood after oral, jejunal or ileal administration ($t_{1/2}$absorption = 2–5 min), reaching tmax at 10–30 min and Cmax of 5.5–18.9 µg/ml of plasma (Figure 5). TC002 was removed from the circulation with a plasma $t_{1/2}$ α of 24–66 min resulting in almost no circulating radiolabel after 4 hr. More ^{3}H-TC002 was absorbed through the ileal and jejunal routes than through the oral route. The absolute bioavailability was 14.9% ± 3.7 SEM and 10.3% ± 1.6 SEM, after ileal and jejunal administration, respectively, compared to 6% ± 1.4 SEM after oral administration. However, over 85% of the radioactivity remained in the intestine and was subsequently recovered in the feces.

Radioactive TC002 was rapidly removed from the body, regardless of the route of administration. By 12 hours, 75% of the urine associated radiolabel was removed, whereas 90% of the fecal associated material was eliminated by 24 hr. Negligible amounts (less than 0.1% of the injected radiolabel) remained

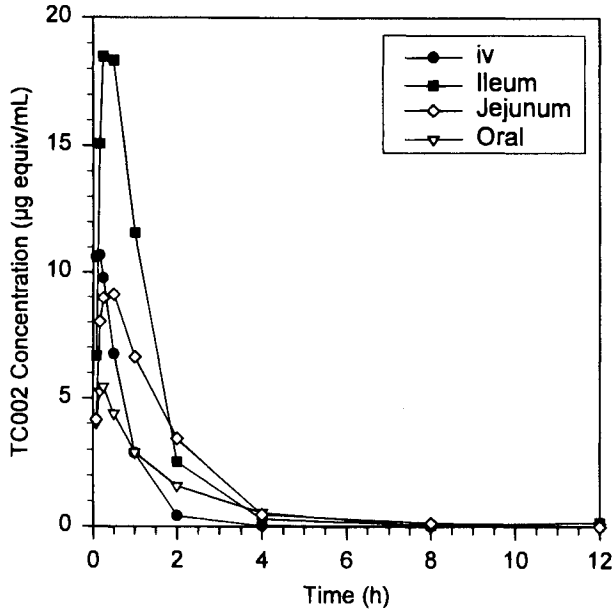


Fig. 5. Plasma concentrations of ^{3}H-TC002 in rats following either *iv*, oral, ileal or jejunal administration. Intestinal and oral doses of TC002 were 100.5 mg/kg (54 mM) and the *iv* dose was 5mg/kg. Mean of 4 rats.

in the intestines, stomach, liver or the bile duct, and less than 0.3% was associated with the carcass 72 hours after oral administration.

Metabolism of TC002

TC002 is metabolized to one major HPLC product. In feces, where 80–85% of the TC002 is directed, the conversion was incomplete and a 60:40 mixture of TC002 and the metabolic product was observed 24 hours post administration. In contrast, urine and plasma contained virtually no intact TC002 at any time point. In plasma and in urine metabolic conversion is complete (>95%) at the earliest sampling points of 5 min and 4 hr, respectively. Similarly rapid conversion of TC002 in plasma occurs *in vitro* at 37°C.

The major metabolic product co-migrates on HPLC with C-24 carboxylic acid TC002 derivative and the C-24 glycine conjugate of TC002. Since our HPLC conditions could not distinguish between these two types of polar analogs, the product generated in animals could consist of either form or a mixture. It is likely that the C-24 methyl ester is being cleaved by esterases found in plasma and the intestine to form this metabolic product.

DISCUSSION

The novel glycosteroid TC002 is clearly effective in the transport of gentamicin when it is admixed with the drug and administered either into the ileal or jejunal intestinal segments, or by oral gavage. A dramatic increase in both plasma levels and absolute bioavailability is achieved in both rats and dogs, even with relatively modest doses of TC002. When formulated with TC002, gentamicin is absorbed quickly and is maintained in the circulation at therapeutic levels (above 4 μg/ml) for prolonged periods of time, returning to baseline within 4 hours in dogs. This pharmacokinetic profile is very similar to the desired clinical profile following *iv* or IM administration. Desirable Cmax levels of gentamicin in patients are between 4 and 6 μg/ml, with the trough between 1–2 μg/ml and a $t_{1/2}$ of 2–4 hours.

Our studies provide some suggestions and raise many questions about mechanisms involved in TC002 action. The distal ileum appears be an optimal site of for absorption of gentamicin in the presence of TC002. There are several possible explanations for this preference. The ileum is slightly alkaline, whereas the other compartments are either highly acidic or more neutral in pH, and the ileum is relatively free of lipids, enzymes and micro-organisms. These factors may interfere with membrane interaction or destabilize aggregation between TC002 and gentamicin. While we have no direct evidence of the formation of aggregates, previous studies have shown that the bis-glycosylated molecules form micelles in water at unexpectedly low concentrations (8) and self-associate more readily than their nonglycosylated counterparts (15).

In addition, the ileum contains an active transport system for bile acids (16,17) and the system is quite permissive for a variety of modified steroids (18). Our data shows that some TC002 is transferred into the circulation following oral, ileal or jejunal administration and that this transfer occurs most efficiently following ileal and jejunal administration. Further studies are needed to specifically address whether TC002 is actively transported.

Alternatively, the distal ileum may be an optimal site of administration because of its proximity to the colon. Preliminary results suggest that gentamicin is also efficiently absorbed when it is co-administered with TC002 into the colon (unpublished observations). Colonic absorption could provide an explanation for the puzzling slow clearance of gentamicin from plasma following intestinal but not *iv* administration. Colonic contents are essentially immobile for several hours and gentamcin/TC002 can therefore have multiple opportunities to penetrate the mucosa, resulting in a prolonged period of absorption that would mask the normally rapid clearance of gentamicin.

It is unlikely that the superior transport capability of TC002 results from a greater ability to seriously disrupt membranes. While the most effective natural bile acids appear to be also damaging to membranes, TC002 is much better tolerated by erythrocyte membranes than taurocholate (8) and the two are similarly cytotoxic for epithelial cells and mucosa.

We have described the effective action of a glycosylated steroid for absorption of a highly polar, non-bioavailable compound. TC002 is a broadly enabling agent for the transport of an aminoglycoside, peptides as well as other small molecule drugs (8, unpublished results). Therefore TC002 is being considered for a variety of therapeutic applications, where there is a need for an oral or nasal delivery alternative to parenteral administration.

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