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Biochemical Pharmacology 64 (2002) 185–190

Biochemical Pharmacology

Renal organic cation and nucleoside transport

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Received 20 September 2001; accepted 7 January 2002

Abstract

We previously reported that the rat organic cation transporter rOCT1 could transport the nucleoside analog deoxytubercidin (dTub) (Chen R, Nelson JA. Biochem Pharmacol 2000;60:215–9). The cationic form of dTub ($dTub^+$) appeared to be the true substrate of rOCT1. We also reported that although rOCT2 is similar to rOCT1, it does not transport dTub at pH 7.4. In this study, we measured the K_m and V_{max} values of $dTub^+$ uptake at a reduced pH (pH 5.4) for both rOCT1 and rOCT2. The difference in substrate activity appears due, in large part, to a poor affinity of rOCT2 for $dTub^+$. The transport efficiency estimated by V_{max}/K_m values for rOCT2 was only 6% that of rOCT1. Chimeras constructed between rOCT1 and rOCT2 revealed that the difference in dTub binding lies within transmembrane domains 2–7. To evaluate the potential of OCT1 in the renal secretion of dTub, tissue distribution and urinary excretion of dTub in *OCT1* knockout mice were measured. No significant difference was observed in renal elimination, plasma level, and tissue distribution of dTub between the knockout and the wild-type mice. Therefore, dTub is a good substrate for OCT1; however, OCT1 does not appear to be necessary for its renal secretion.

Keywords: Organic cation transporter; Knockout mice; Deoxytubercidin; Chimeric construction; Kinetics; Nucleoside analog

1. Introduction

Mammalian kidneys serve to maintain an extracellular environment compatible with life by processes of filtration, reabsorption, and secretion. Two major secretory systems assist in the elimination of many positively and negatively charged toxins, i.e. the OCT and OAT systems, respectively. Although a number of nucleoside transporters are known [1], we have determined that an OCT is responsible for the renal secretion of the model nucleoside, dTub [2,3]. Specifically, dTub uptake by mouse kidney slices is inhibited by organic cations but not organic anions, and, conversely, dTub inhibits the uptake of organic cations but not organic anions by the slices [2]. Additionally, the renal secretion of dTub in mice is inhibited by the OCT inhibitor cimetidine [3]. We recently reported [4] that the rat organic cation transporter rOCT1 utilizes dTub, whereas the similar rOCT2 does not. Further, the cationic form of dTub ($dTub^+$) appeared to serve as the true substrate since enhanced transport was observed with decreasing pH. Consequently, we report herein the relative K_m and V_{max} values for dTub for both rOCT1 and rOCT2 by studying the transport at reduced pH (pH 5.4). The different substrate activity appears due largely to a poorer affinity of rOCT2 for $dTub^+$.

rOCT1 and rOCT2 belong to the major facilitator superfamily, members of which include bacterial drug resistance proteins, proton solute symporters, and facilitative diffusion systems for sugars [5,6]. They are characterized by a similar secondary structure with twelve transmembrane-spanning α-helix motifs. Besides their similar secondary structure, both rOCT1 and rOCT2 are located on the basolateral membrane of epithelial cells, share broad substrate specificity, and mediate the first step in the elimination of various compounds at the liver and kidney by facilitated diffusion [7]. It is therefore rational to assume that rOCT1 and rOCT2 handle their substrates in a very similar way. The dramatic difference between the transport of dTub between these two transporters allows us to explore the structural features involved in substrate translocation. Herein, three sets of chimeras of rOCT1 and

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Abbreviations: dTub, deoxytubercidin; OAT, organic anion transporter; OCT, organic cation transporter; TEA, tetraethylammonium.

0006-2952/02/$ – see front matter
PII: S0006-2952(02)01062-6

rOCT2 were constructed to identify the domains in rOCT1 responsible for the specific uptake of dTub.

To evaluate the potential role of OCT1 in the renal elimination of dTub, we measured the tissue distribution and urinary excretion of [^{3}H]-dTub in *OCT1* knockout mice. Although the tissue distribution of the classical OCT substrate TEA was altered by genetic elimination of OCT1 [8], the renal elimination, plasma level, and tissue distribution of dTub were the same as that observed in wild-type mice. In summary, dTub is a good substrate for OCT1; however, OCT1 does not appear to be necessary for its renal secretion.

2. Materials and methods

2.1. Materials

[^{14}C]-TEA (2.4 to 3.36 mCi/mmol) was purchased from DuPont NEN. [^{3}H]-dTub (9.3 Ci/mmol) was prepared by Moravek Biochemicals Inc., using dTub synthesized in our laboratory as previously described [9]. Methoxyflurane was from Medical Developments Australia Pty. Ltd. Other chemicals were obtained from the Sigma Chemical Co.

2.2. Methods

2.2.1. Xenopus laevis oocyte injection and transport measurements

The rOCT1 [10], rOCT2 [11], and mouse OCT1 (mOCT1) [12] cDNAs in the pSPORT1 vector (Life Technologies, Inc.) were gifts of Dr. Vadivel Ganapathy (Medical College of Georgia), Dr. John B. Pritchard (Laboratory of Pharmacology and Chemistry, National Institute of Environmental Health Sciences), and Dr. Richard Green (University of Illinois at Chicago College of Medicine), respectively. The 5′-capped cRNAs of the transporters, chimeras, and mutations were synthesized *in vitro* using the Ambion T7 mMessage mMachine transcription kit (Ambion, Inc.) as previously described [4]. *Xenopus laevis* oocytes were collected, defolliculated manually, and injected with 20 ng (40 nL) of cRNA or with an equal volume of water per oocyte. In each experiment, 15 oocytes were used per group. The functions of the injected cRNAs were measured 3 days after injection. For uptake experiments, the oocytes were incubated for 4 hr in modified Barth's Solution containing radiolabeled substrates (5 μCi/mL, 100 μM for [^{3}H]-dTub, and 1 μCi/mL, 420 μM for [^{14}C]-TEA). At the end of the uptake period, the oocytes were washed three times in ice-cold Barth's solution. They were then lysed in 200 μL of lysis buffer containing 0.2% sodium dodecyl sulfate and 0.2 N NaOH, and the radioactivity was determined by liquid scintillation counting. For kinetic determinations, the 1-hr uptake was measured at pH 5.4 for dTub and pH 7.4 for TEA. For each concentration of substrate, a water-injected group was used as an indicator of the endogenous uptake.

2.2.2. Preparation of chimeras

The chimeras were constructed using both the native restriction enzyme sites and the engineered sites introduced through site-directed mutagenesis. The chimeras were confirmed by restriction enzyme analysis and by sequencing. Specifically, both rOCT1 and rOCT2 have a *Sex*AI site at Leu^{141} in the first extracellular loop. rOCT1 has a *Bbr*PI site at His^{368} at the end of transmembrane domain 7. A *Bbr*PI site is created in the corresponding site in rOCT2. rOCT2 has an *Nco*I site at Ile^{445} within transmembrane domain 9. An *Nco*I site was introduced into rOCT1 at the corresponding position. Three sets of chimeras were made by switching the DNA fragments between these restriction enzyme sites.

2.2.3. Site-directed mutagenesis of amino acid 145

The Transformer™ Site-Directed Mutagenesis Kit (CLONTECH Laboratories, Inc.) was used to generate mutations rOCT1D145H and rOCT2H145D. The selection primer (5′-GTTGGTGCGGAGATCTCGGTAG-3′) transfers the *Eco*RV site in the pSPORT1 vector into a *Bgl*II site (underlined). The mutation primer for rOCT1D145H is 5′-CCTGGTGTGTGGACACGCCTGGAAAGTG-3′, and the mutation primer for rOCT2H145D is 5′-CCTGGTGTGTGCTGACTCCTGGATGCTG-3′. The mutations were confirmed by restriction enzyme digestion and by DNA sequencing.

2.2.4. Distribution and renal elimination of dTub in OCT1 knockout mice

The *OCT1*$^{-/-}$ and wild-type mice were generated as previously described [8]. dTub was administrated intravenously at a dose of 1 mg/kg into the tail veins of mice lightly anesthetized with methoxyflurane. Twenty minutes later, the mice were killed, and blood was collected by axillary bleeding. At the termination of the experiment, urine was collected from the bladder, and organs were removed and homogenized in a 4% (w/v) bovine serum albumin solution. The amount of dTub was determined in the homogenates as previously described [8,13].

2.2.5. Sequence analysis

DNA sequencing of the chimeras and mutations was performed by the DNA Sequencing and Analysis Facility Core in this institution. Sequence compare and alignments were performed by Wisconsin Package Version 10.1, Genetics Computer Group (GCG).

2.2.6. Data analyses

Statistical analyses were performed using the StatMost™ statistical analysis and graphics program (Dataxiom Software Inc.). Significance of differences was determined by the unpaired Student's *t*-test. The K_m and V_{max} values were

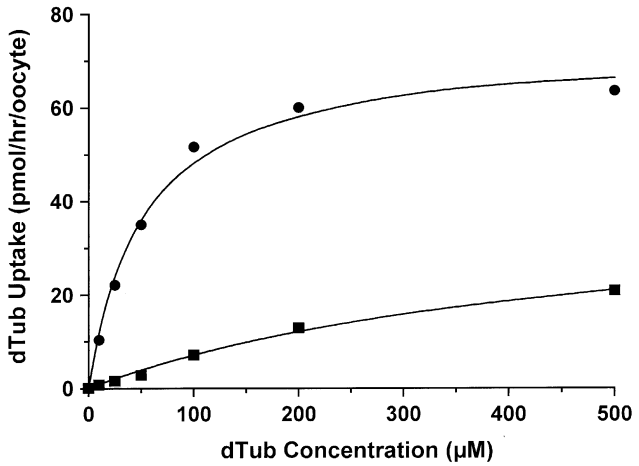

Fig. 1. Uptake of [^{3}H]-dTub into *X. laevis* oocytes injected with the cRNA of rOCT1 or rOCT2. Oocytes were injected with 20 ng of rOCT1 (●) or rOCT2 (■) cRNA or an equal volume of water. Three days after injection, the 1-hr uptake of [^{3}H]-dTub (5 μCi/mL, 10, 20, 50, 100, 200, and 500 μM) was measured at pH 5.4. Mean uptake values for 15 oocytes per group are shown after subtraction of water-injected controls.

determined by fitting the data into the Michaelis equation [14] by non-linear regression.

3. Results and discussion

3.1. Relative affinities of rOCT1 and rOCT2 for dTub

We previously reported that dTub was not transported by rOCT2 in the oocyte expression system. However, when the pH in the uptake solution was lowered from 7.4 to 5.4, a 2-fold increase of uptake compared with water-injected controls was observed (data not shown). Consequently, subsequent experiments were performed at pH 5.4 to increase the dTub$^+$, the putative true substrate [4]. Since the pK_a value for dTub is 5.3 [2], approximately 50% of dTub is ionized at this pH. The transport of dTub into *X. laevis* oocytes by rOCT1 and rOCT2 is illustrated in Fig. 1. The calculated K_m values for dTub$^+$ in this experiment were 23 and 212 μM for rOCT1 and rOCT2, respectively, and the corresponding V_{max} values were 73 and 41 pmol/hr/oocyte (Table 1). The transport efficiency estimated by the V_{max}/K_m values for TEA was approximately the same for rOCT1 and rOCT2 (1.6 and 1.8, respectively; Table 1); however, the dTub V_{max}/K_m value for rOCT2 was only about 6% of that of rOCT1 (0.2 vs 3.2; Table 1). This is in sharp contrast to most of the organic cations that have been evaluated as substrates or inhibitors of rOCT1 and rOCT2, i.e. the affinities of the two carriers are generally similar [7]. Although these results are discussed with regard to the dTub substrate activity of rOCT1 and rOCT2, it is possible that lowering the external pH may have effects on these transporters that might lead to a different interpretation.

dTub and TEA mutually and competitively inhibit the transport of the other [4], suggesting that they share a common site on rOCT1. Thus, dTub appears to be a useful tool to probe the active site of organic cations for OCTs. rOCT1 and rOCT2 are approximately 68% identical in amino acid sequence; therefore, we prepared chimeras in an attempt to locate the active site as has been successfully applied for some nucleoside transporters [15,16]. Functional activities of the chimeras were determined by expression in the frog oocyte system as illustrated above. The relative uptake of dTub was compared with that of TEA, i.e. the goal was to identify the region of these OCTs responsible for their differential affinities for dTub compared with TEA. Fig. 2 illustrates the three sets of chimeras and their relative functions. Chimeras AAAB and BBBA have the two and one-half transmembrane domains at the C-terminus exchanged. The function of AAAB is similar to that of rOCT1, and the function of BBBA is similar to that of rOCT2. Therefore, the dTub binding site does not appear to lie within the two transmembrane domains at the C-terminus. Chimera AABB functions more closely to rOCT1 and BBAA is close to rOCT2, indicating that the dTub binding is located within the seven transmembrane domains at the N-terminus. However, replacing transmembrane domains 2–7 of AABB with the corresponding fragment from rOCT2 significantly reduced the uptake of dTub by this chimera (ABBB), and gaining the first transmembrane domain from rOCT1 did not make chimera ABBB significantly different from rOCT2. We concluded from the chimera studies that the transmembrane domains 2–7 are critical for rOCT1 to transport dTub. Chimera BAAA was not functional, perhaps due to improper helical packing or unstable protein structure.

Table 1
Comparing the K_m and V_{max} or rOCT1 and rOCT2 for the uptake of TEA and dTub

Substrate	rOCT1			rOCT2		
	K_m (μM)	V_{max} (pmol/hr/oocyte)	V_{max}/K_m	K_m (μM)	V_{max} (pmol/hr/oocyte)	V_{max}/K_m
dTub$^+$	23	73	3.2	212	41	0.2
TEA	93	148	1.6	125	226	1.8

K_m and V_{max} were determined using the *Xenopus* oocyte expression system. Uptake of[^{3}H]-dTub and [^{14}C]-TEA into the oocytes was measured 3 days after injecting the cRNA for rOCT1 or rOCT2 as described in Section 2. Incubations were performed for 1 hr at pH 5.4 for dTub and pH 7.4 for TEA. The K_m and V_{max} values were determined by line fitting into the Michaelis equation using non-linear regression analysis in the StatMost™, statistical analysis and graphics program (Dataxiom Software Inc.). The K_m and V_{max} values for dTub represent the ionized form i.e. dTub$^+$.

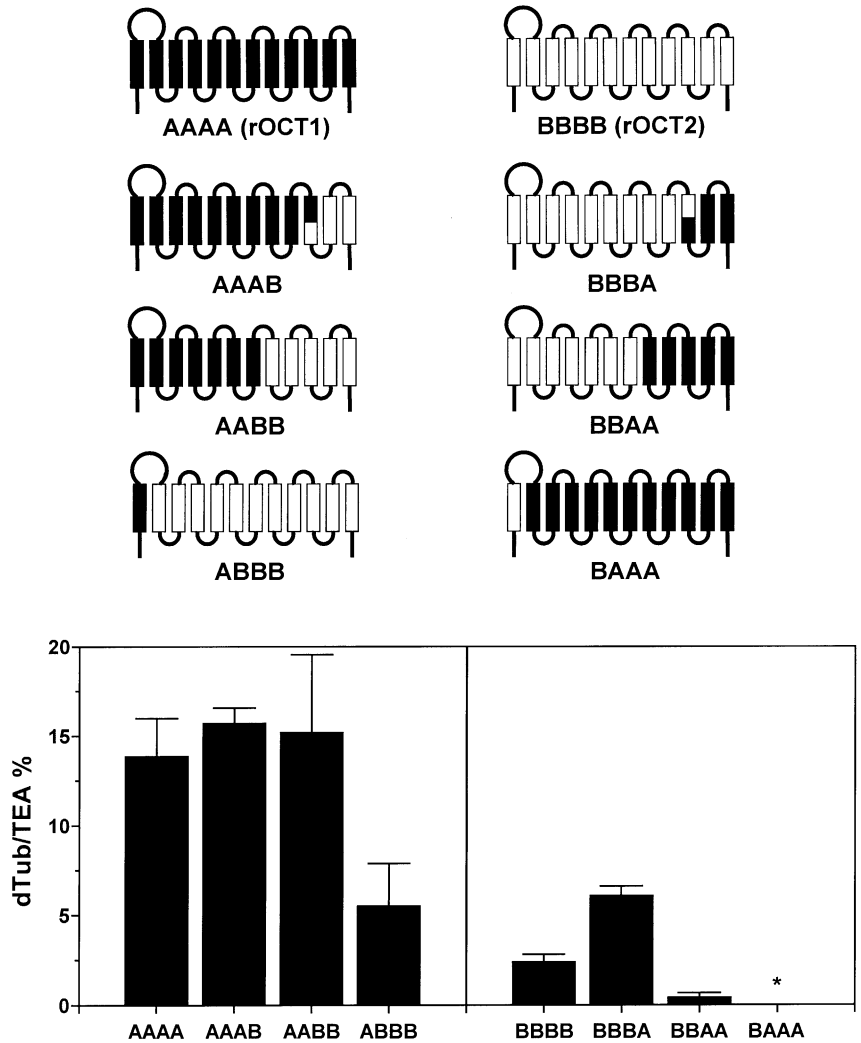

Fig. 2. Chimeras of rOCT1 and rOCT2 and their uptake of dTub compared with that of TEA. Chimeras were constructed as described in Section 2 and were named according to the origin of the DNA fragments. AAAA stands for rOCT1, and BBBB stands for rOCT2. The chimeras were expressed in *Xenopus* oocytes. The 4-hr uptake of TEA (at pH 7.4) and of dTub (pH 5.4) was measured 3 days after injection. After subtracting the uptake of water controls, the uptake of dTub as a percentage of TEA was calculated for each chimera and is presented in this figure. Data represent the means ± SEM of at least three individual experiments with 10–15 oocytes per group. Key: (*) no detectable activity for this chimera.

Dwyer [17] proposed the structure of GLUT3 as the twelve transmembrane helices forming a barrel with a central hydrophilic pore. Zeng *et al.* [18] predicted that five transmembrane helices of GLUT1 line a substrate passage channel. We could infer that for OCTs, a network of negatively charged, conserved amino acids line the pore region, which facilitates the binding and translocation of organic cations. From our studies with dTub, it is likely that there are some negatively charged amino acids in rOCT1 that lie within the pore region and bind or stabilize dTub, or form a cavity of the size for dTub. In rOCT2, however, a lack of these critical sites renders it inefficient to transport dTub. There are 228 amino acids in transmembrane domain 2–7, and 14 of them are negatively charged (aspartic acid or glutamic acid). We compared the amino acid sequence of OCT1s and OCT2s from pigs, humans, mice, and rats. For these OCTs, amino acid 145 in OCT1s was invariably negatively charged aspartic acid (D), whereas it was neutral (in hOCT2 and pOCT2) or positively charged (rOCT2 and mOCT2) in the OCT2s. Thus, we prepared mutation rOCT1D145H in which Asp^{145} in rOCT1 was changed to histidine; and mutation rOCT2H145D in which His^{145} of rOCT2 was changed to aspartic acid by site-directed mutagenesis. The mutants were expressed in oocytes. There was no significant difference between the mutants and their parental transporters in the uptake of [^{14}C]-TEA (1 μCi/mL, 420 μM, pH 7.4) or [^{3}H]-dTub (5 μCi/mL, 100 μM, pH 5.4), conditions at which the differential uptake of dTub by rOCT1 and rOCT2 is easily observed. Thus, this alteration of amino acid 145 does not change the differential transport of rOCT1 and rOCT2 for dTub.

3.2. Role of OCT1 in the renal secretion of dTub

The critical experiment to evaluate the role of this carrier in the renal elimination of dTub would be to determine

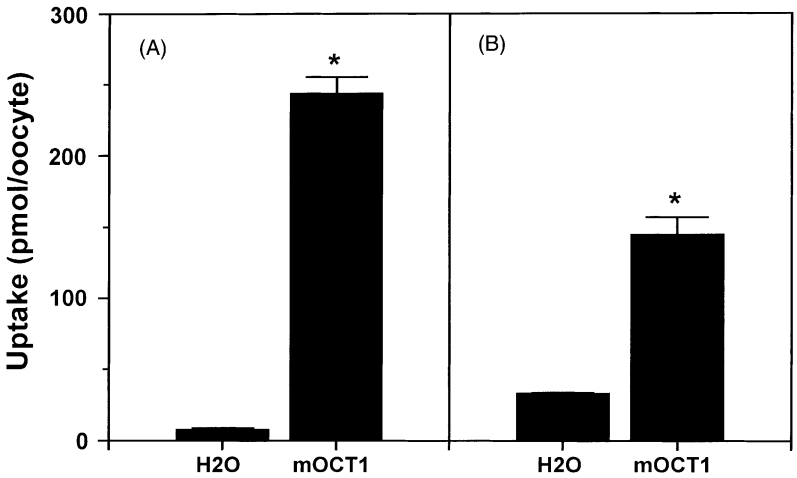

Fig. 3. Uptake of [^{14}C]-TEA and [^{3}H]-dTub into *X. laevis* oocytes injected with the cRNA of mOCT1. Oocytes were injected with 20 ng of cRNA or an equal volume of water. Three days later, the 4-hr uptake of (A) [^{14}C]-TEA (1 μCi/mL, 420 μM) or (B) [^{3}H]-dTub (5 μCi/mL, 100 μM) was measured at pH 7.4. Mean values ± SEM for 10–15 oocytes per group are shown. Key: (*) significantly different ($P < 0.05$) from water-injected controls, by Student's *t*-test.

urinary excretion of dTub in mice lacking OCT1. In preparation for such an experiment, we first determined that the mOCT1 utilized dTub as a substrate. Fig. 3 illustrates that mOCT1 utilizes both TEA and dTub. Since mOCT1 is 95% identical in amino acid sequence to rOCT1, this is an expected finding. We next determined the tissue distribution and urinary elimination of dTub in *OCT1* knockout mice (*OCT1*$^{-/-}$ mice) 20 min after intravenous administration of radiolabeled dTub. dTub is not metabolized extensively in mice [2], so the radioactivity values are a good reflection of the true distribution of dTub. Data in Table 2 demonstrate that dTub is secreted primarily by the kidneys. Approximately 50% of the injected dTub was eliminated in the urine after 20 min. Loss of OCT1 did not significantly change the plasma level, tissue distribution, or urinary excretion of dTub, an observation that strongly suggests that this carrier alone is not necessary for the renal secretion of dTub. On the other hand, the *OCT1* knockout mice are profoundly deficient in their uptake of TEA by the liver [8]. The lack of effect of loss of OCT1 suggests that OCT1 is not necessary for the renal elimination of dTub, i.e. either the carrier is not involved or other transporters compensate for its loss. In this regard, Jonker *et al*. [8] reported that expression of OCT2 and OCT3 is not increased in *OCT1*$^{-/-}$ mice, indicating that loss of OCT1 is not compensated for by up-regulation of OCT2 or OCT3. Although dTub is not a good substrate for rOCT2 as shown above, we do not know whether this is true for mOCT2. The transport may be compensated for by other transporters such as the OCTNs or nucleoside transporters. It is also possible that OCT1 is not rate-limiting for the renal secretion of dTub. That is, dTub may have sufficient lipid solubility enabling it to passively diffuse through the basolateral membrane such that transport at this site is not necessary for its secretion. Perhaps the movement of dTub out of the proximal tubule cells at the apical membrane is the rate-limiting step, the postulated site for active transport of organic cations in their renal secretion.

In summary, chimeric studies located transmembrane domains 2–7 for the specific transport of dTub in rOCT1. Although dTub is a good substrate for OCT1, this carrier does not appear necessary for the renal elimination of dTub in mice. This finding suggests that OCT1 is either not rate-limiting in the process of renal secretion of dTub or that other carriers compensate for the absence of OCT1 expression.

Table 2
Plasma level and tissue distribution of dTub (as a concentration or a percentage of dose) 20 min after intravenous administration in *OCT1* knockout mice

	Wild-type	*OCT1*$^{-/-}$	Ratio (*OCT1*$^{-/-}$/wild-type)	*P* value
Plasma (ng/mL)	486 ± 171	491 ± 121	1.01	0.96
Small intestine (ng/g)	27 ± 3	26 ± 6	0.96	0.79
Contents small int. (%)	0.57 ± 0.17	0.81 ± 0.21	1.42	0.12
Liver (%)	0.81 ± 0.12	1.08 ± 0.17	1.32	0.05
Kidneys (%)	0.22 ± 0.12	0.20 ± 0.03	0.92	0.80
Urine (%)	57 ± 6	48 ± 10	0.84	0.17

[^{3}H]-dTub was injected into the tail veins of mice at a dose of 1 mg/kg. Twenty minutes after injection, the mice were killed, and blood and urine were collected. Organs were removed and homogenized. Levels of radioactivity in the homogenates were determined as described in Section 2. Four mice were used in each group. Data represent means ± SD. *P* values were determined by the unpaired Student's *t*-test.

Acknowledgments

Work by J.W. Jonker was supported, in part, by a grant from the Dutch Cancer Society (NKI 97-1434) to Dr. Alfred H. Schinkel. This work was also supported by National Institutes of Health Grant DK41606, the Institute for Digestive Diseases and Kidney, and by 2-P30-CA16672, a National Cancer Center Core Grant. In partial fulfillment of the requirements for the Ph.D. Degree for Rong Chen in The University of Texas, Graduate School of Biomedical Sciences at Houston.

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