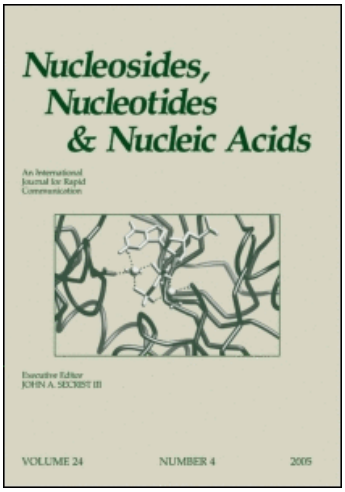

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P-Glycoprotein and the Renal Secretion of Deoxyribonucleosides

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P-GLYCOPROTEIN AND THE RENAL SECRETION OF DEOXYRIBONUCLEOSIDES

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ABSTRACT

2'-Deoxytubercidin is secreted by the organic cation secretory system of mouse kidney. The presented data indicate that this secretion occurs by a transport system other than the MDR1 P-glycoprotein.

INTRODUCTION

In addition to filtration by the glomerulus, human[1] and mouse[2] kidneys are capable of reabsorbing adenosine (Ado) and secreting deoxyadenosine (dAdo). We have utilized 2'-deoxytubercidin (dTUB) as a model to study the mechanisms for dAdo renal secretion since, unlike dAdo, dTUB is neither toxic nor metabolized[3]. Secretion of dTUB by mouse kidney occurs via the classical renal secretory system for organic cations (OCT). Specifically, the tissue-specific and energy-dependent uptake of radioactive dTUB by mouse kidney slices is inhibited by tetraethylammonium (TEA), N^1-methylnicotinamide (NMN) and choline (substrates for OCT) but not by p-aminohippurate (PAH), probenecid or phenol red (substrates for the organic anion secretory system). Reciprocally, dTUB inhibits active TEA but not PAH uptake by the slices[3]. Further, dTUB renal secretion in mice is prevented by cimetidine, a relatively selective inhibitor of OCT[2]. P-glycoprotein (Pgp), the membrane protein responsible for enhanced drug efflux in multidrug resistant (MDR) tumor cells[4], is normally expressed in the apical membranes of renal proximal tubule cells[5] (the postulated site of active OCT[6]). Since a number of Pgp substrates are lipophilic organic cations, we hypothesized that Pgp is the carrier for OCT in mammalian kidney[7]. We report herein that transepithelial diffusion of dTUB is not enhanced in epithelial cells overexpressing Pgp, that dTUB and several ribo- and deoxyribo- nucleosides do not inhibit the enhanced flux of cimetidine and daunomycin in these cells, and that several purine and pyrimidine nucleosides are equally toxic to wild-type and MDR cell lines. Taken together, these observations suggest that dTUB and other nucleosides may be useful substrates to identify an OCT system other than Pgp.

MATERIALS AND METHODS

Materials. [^{3}H]-dTUB (16 Ci/mmole) was custom synthesized by and [^{3}H]-azidothymidine (33 Ci/mmole) was purchased from Moravek Biochemicals (City of Industry, CA). [^{3}H]-Cimetidine (25 Ci/mmole) was purchased from Amersham Corp. (Arlington Heights, IL) and [^{3}H]-daunomycin (1.3 Ci/mmole) was obtained from New England Nuclear (Boston, MA). [^{14}C]-Inulin (3.74 μCi/mg) was purchased from ICN Radiochemicals (Irvine, CA). Type I collagen, tubercidin (TUB), adenosine (Ado), deoxyadenosine (dAdo), deoxycoformycin (DCF), 3'-azido-3'-deoxythymidine (AZT), cimetidine, daunomycin, verapamil, and quinidine were purchased from Sigma Chemical Company (St. Louis, MO). dTUB was synthesized and generously provided to us by Dr. Morris Robins[8]. 2-chloro, 2'-deoxyadenosine (2 CldAdo) and 2-fluoro-9-(β-D-arabinofuranosyl)adenine 5'-monophosphate (FaraAMP) were obtained from the Drug Synthesis and Development Branch of the National Cancer Institute. Cyclopore membrane inserts were the products of Becton Dickinson Labware (Lincoln Park, NJ).

Cell Culture. The MDCK cells were maintained in Dulbecco's Modified Eagle medium (D-MEM) containing 25 mM glucose, 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. The MDR-MDCK cells were derived by transfecting wild-type MDCK cells with a retrovirus carrying the human multidrug-resistance (MDR1) cDNA[9] and maintained in D-MEM medium with 1.28 μg/ml colchicine. Both MDCK and MDR-MDCK cell lines were generously provided by Dr. Michael Gottesman of the National Cancer Institute. The CHO and CHO/Adr cells are wild-type and adriamycin-resistant Chinese hamster ovary cells, respectively, that were maintained in McCoy's 5A medium. CHO/Adr cells overexpress pgp1 gene, the Chinese hamster homolog of the mouse mdr3 gene[10], and have been cultured in medium with 0.5 μg/ml adriamycin. Cl 1D and Cl 1D/VCR cells are wild-type and vincristine-resistant murine fibroblasts that were maintained in high glucose D-MEM medium. Cl 1D/VCR cells have an amplification of the mdr1 gene[11], and have been maintained in D-MEM medium with 0.2 μg/ml vincristine. Cl 1D, Cl 1D/VCR and CHO/Adr cells were provided to us by Dr. Macus Tien Kuo of the Department of Molecular Pathology, University of Texas M.D. Anderson Cancer Center. All drug selecting agents were removed from the medium of resistant cell lines during performance of the experiments. The cloning efficiency of the cells was determined by exposure of cells to various concentrations of drugs for seven days as described by Drake, et al.[12].

Transepithelial Transport. The transepithelial transport of [^{3}H]-dTUB [^{3}H]-daunomycin, [^{3}H]-cimetidine and [^{3}H]-azidothymidine across MDCK and MDR-MDCK cell monolayers was determined as described by Horio, et al.[13]. The Cyclopore membrane inserts (25 mm with 0.45 μm pores) were coated with 1 ml of 100 μg/ml Type I collagen in 0.1 N acetic acid. Cells were cultured on these precoated membrane inserts at 37°C for five days and then preincubated in fresh medium with or without inhibitors for 30 min. Tritium labeled compounds (2 μM; 0.1 μCi/ml) with ^{14}C-inulin (2 μM; 0.025 μCi/ml), with or without inhibitors, were added to either basolateral or apical sides of the monolayers.

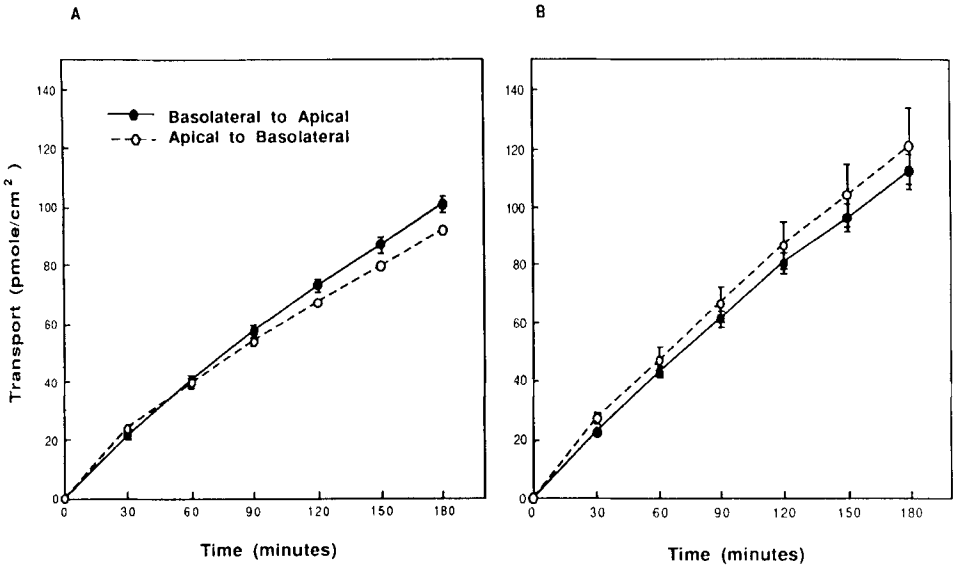

FIG. 1. Transepithelial flux of dTUB across MDCK and MDR-MDCK cell monolayers. MDCK (A) and MDR-MDCK (B) cells were cultured on collagen coated filter inserts for five days. ^{3}H-dTUB (2 μM) with ^{14}C-inulin (2 μM) was added to either the basolateral (●) or apical (o) side of the monolayer. Samples from the opposite side were measured at 30 min intervals for 3 hr as described in Materials and Methods. The values of ^{3}H-dTUB transport have been corrected for the amounts of ^{14}C-inulin which passed to the opposite side by paracellular pathways. The results shown are the mean values ± S.E. of three separate experiments.

Samples of 1 ml of medium from the reciprocal side were removed at 30 min intervals to determine radioactivity and an equal volume of fresh medium, with or without inhibitors, was added back to replace that sampled. The amounts of [^{3}H]-labeled solutes transported to the reciprocal side were corrected by the amounts of internal standard, [^{14}C]-inulin, leaking to that side. Transport velocity of daunomycin, cimetidine and AZT was determined by linear regression analysis of the cumulative flux across the cell monolayer as a function of time. The velocity of dTUB flux was calculated by the value observed at 30 minutes.

RESULTS

The MDCK cells are derived from a dog kidney distal tubule cell and are useful to study transcellular transport in culture because they grow on porous membranes in a polarized fashion, i.e., the basolateral membrane attaches to the membrane surface leaving the apical side of the cell exposed to the culture medium[14]. MDR-MDCK cells have been retrovirally-transduced to express the human form of MDR1[9]. Analogous to proximal tubule cells, the P-

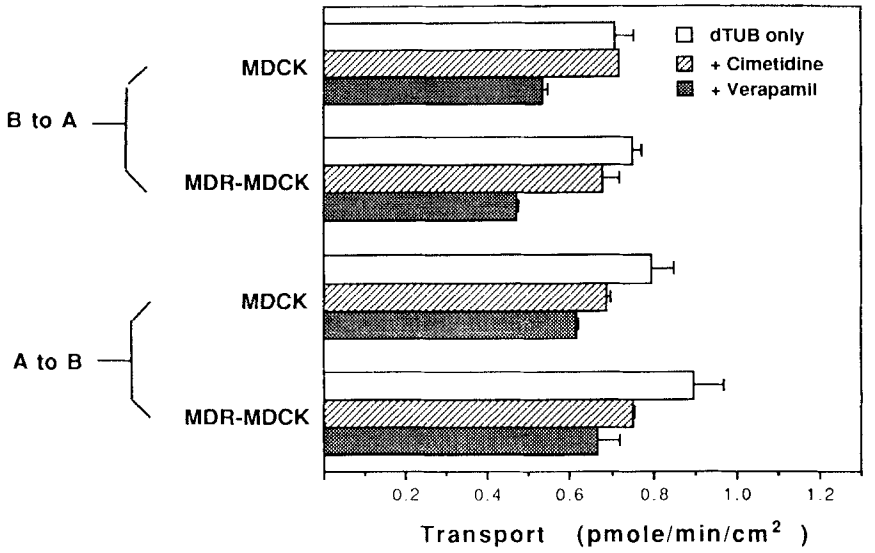

FIG. 2. Effects of cimetidine and verapamil on transepithelial flux of dTUB across MDCK and MDR-MDCK cells. Polarized MDCK and MDR-MDCK monolayers were preincubated in fresh medium (□) or in medium containing 200 μM cimetidine (▨) or 20 μM verapamil (■) for 30 min at 37°C. Then ^{3}H-dTUB (2 μM) with ^{14}C-inulin (2 μM) was added and transepithelial transport was determined as described in the legend to Fig. 1. The results shown are mean values ± S.E. of three separate experiments.

glycoprotein is expressed on the apical membrane of MDR-MDCK cells, and these cells have an increased capacity to transfer MDR drugs (i.e., vinblastine, daunomycin) from the basolateral-to-apical medium, compared to the flux observed in MDCK cells and compared with the apical-to-basolateral flux[13]. The transepithelial flux of dTUB across MDCK cells is not increased by the over-expression of Pgp (Figure 1). Specifically, the flux is approximately identical for MDCK and MDR-MDCK cells (Figure 1A versus 1B) whether the vectorial component is basolateral to apical or vice versa. This is in sharp contrast to the flux observed in these same cells when either cimetidine (unpublished observation) or daunomycin[13] is used as substrate. That is, the basolateral-to-apical flux in the MDR-MDCK cells for these compounds is at least 3-fold that observed in the MDCK cells over a wide range of substrate concentration. Further evidence that dTUB is not a substrate for Pgp is provided in the data illustrated in Figure 2.

Specifically, cimetidine (a substrate for Pgp) and verapamil (an inhibitor of Pgp) do not alter dTUB flux by MDCK cells in a manner consistent with its transport by Pgp. That is, these agents only slightly inhibit the flux in either direction, and the degree of inhibition is relatively independent of the Pgp expression (MDCK versus MDR-MDCK cells). The Pgp

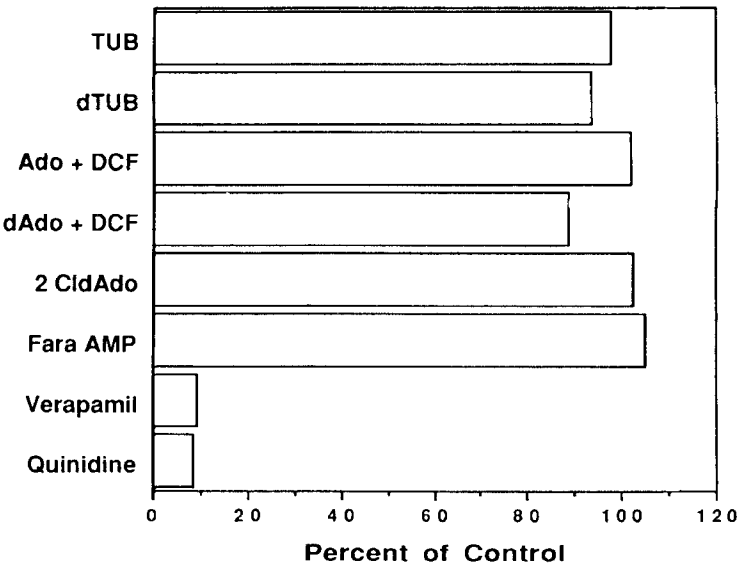

FIG. 3. Failure of purine nucleosides to inhibit the transepithelial transport of cimetidine by MDR-MDCK cells. MDR-MDCK cells were cultured on collagen coated filter inserts for five days. Before adding ^{3}H-cimetidine (2 μM) and ^{14}C-inulin (2 μM), the cells were preincubated in either fresh medium or in medium containing 200 μM purine nucleosides, 200 μM verapamil, or 200 μM quinidine, for 30 min at 37°C. The concentration of the adenosine deaminase inhibitor 2'-deoxycoformycin (DCF) was 1 μM. Transepithelial transport of cimetidine was determined by counting the radioactivity of aliquots removed from the apical side at 30 min intervals as described in Materials and Methods. The transepithelial transport rate of cimetidine in controls was 0.342 ± 0.014 pmole/min/cm^2.

inhibitors verapamil and quinidine inhibit the basolateral-to-apical flux of cimetidine (Figure 3) or daunomycin (Figure 4) in MDR-MDCK cells by at least 80%. On the other hand and under the same incubation conditions, dTUB and several other purine nucleosides are without effect on either cimetidine or daunomycin transepithelial flux in these cells.

The failure of dTUB to serve as a substrate for Pgp is not solely due to its nucleoside characteristic since an enhanced rate of diffusion of AZT is observed (basolateral to apical) in the MDR-MDCK cells compared with the MDCK cells (Figure 5). An increment of apical-to-basolateral over basolateral-to-apical flux is observed in the wild-type cells (Figure 5A), the possible significance of which is unknown.

The MDR phenotype is characterized by cross-resistance among a number of toxic compounds, frequently observed with lipophilic organic cation natural products[15]. Cross-resistance is not generally seen for alkylating agents and antimetabolites. None of six purine or pyrimidine nucleosides tested demonstrated marked cross-resistance in 3 separate MDR cell

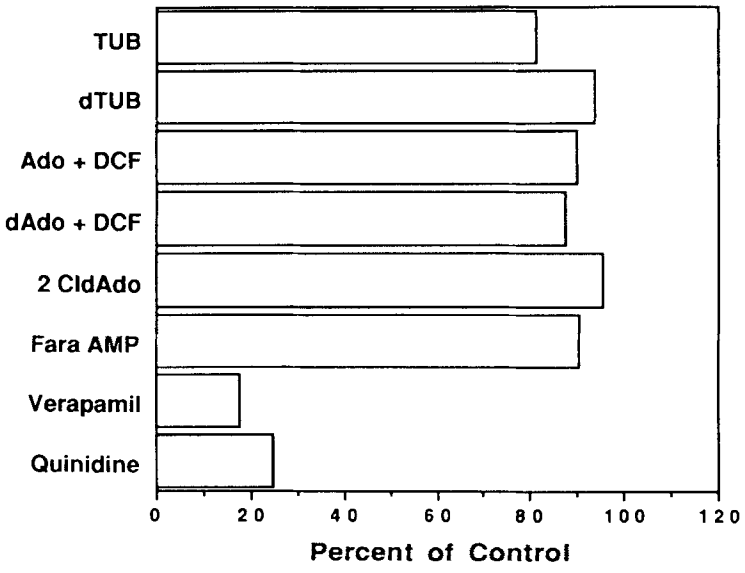

FIG. 4. Failure of purine nucleosides to inhibit daunomycin transport by MDR-MDCK cells. Polarized MDR-MDCK cells were preincubated in medium containing 200 μM purine nucleosides, 200 μM quinidine or 200 μM verapamil at 37°C for 30 min. At that time, ^{3}H-daunomycin (2 μM) with ^{14}C-inulin (2 μM) was added to the basolateral medium and transepithelial transport was determined as described in the legend to Fig. 3. The transepithelial transport rate of daunomycin in controls was 0.809 ± 0.039 pmole/min/cm^2.

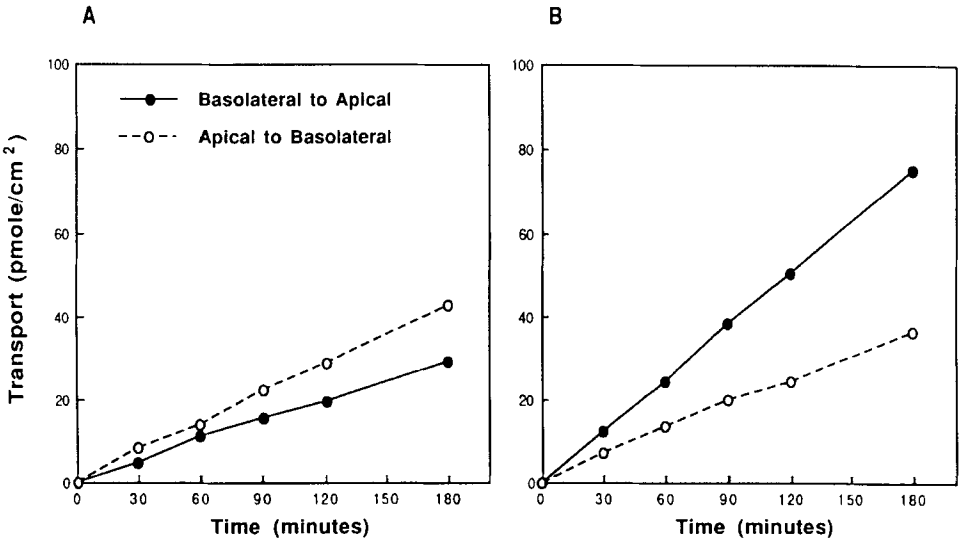

FIG. 5. Transepithelial flux of AZT across MDCK and MDR-MDCK cells. Polarized MDCK (A) and MDR-MDCK (B) cells were preincubated in fresh medium for 30 min. ^{3}H-AZT (2 μM) with ^{14}C-inulin (2 μM) was then added to either the basolateral side (●) or apical side (o) of the monolayer and transepithelial flux was determined as described in Materials and Methods.

TABLE 1. Lack of resistance to selected purine and pyrimidine nucleosides in three different MDR cell lines.

	Cell Line[a]					
Nucleosides	MDCK	MDR-MDCK	CHO	CHO/Adr	Cl 1D	Cl 1D/VCR
Tubercidin	0.024	0.012	0.0073	0.0039	0.0032	0.0031
2 CldAdo	0.012	0.011	0.36	0.47	0.23	0.10
FaraAMP	5.9	12	11	13	11	3.6
Ado + DCF	31	31	68	80	106	192
dAdo + DCF	6.0	11	19	20	17	22
AZT	350	65	1030	1150	2450	2450
MDR Drugs						
Daunomycin	3.1	120	-	-	11	370
Adriamycin	-	-	0.047	10	-	-

[a]The values shown are the concentrations (μM) that inhibit colony formation by 50% of control values during 5 to 7 days incubation[12]. The resistant cell lines used each display the MDR phenotype as described in Materials and Methods.

lines (Table 1). The MDR phenoypte has been clearly established for each of these cells (by measurement of Pgp mRNA and/or protein). The resistance to the MDR drugs daunomycin and adriamycin in Table 1 confirms the phenotype during these experiments.

DISCUSSION

The data reported herein clearly demonstrate that dTUB is not a substrate for Pgp. That is, the transepithelial flux is independent of the degree of Pgp expression in MDCK cells (Figure 1), and dTUB does not inhibit the increased flux of cimetidine (Figure 3) or daunomycin (Figure 4) in these cells. Further, toxic nucleoside analogs of dTUB do not demonstrate cross-resistance in tumor cell lines that possess the characteristic MDR phenotype (Table 1). The basolateral-to-apical flux of AZT, another nucleoside, did appear to be somewhat greater in the MDR-MDCK cells versus the parent cell line (Figure 5). This agent was relatively nontoxic to the MDR cell lines studied, for which no cross-resistance was observed (Table 1). Others have reported cross-resistance to AZT in CEM[16] and K562[17] cells that overexpress Pgp, and AZT does undergo renal secretion in humans, although the secretory

mechanism is unknown[18,19]. Thus, failure of dTUB to serve as a substrate for Pgp is not solely due to its being a nucleoside. Finally, the failure to observe an augmented rate of transepithelial flux in the MDR-MDCK versus MDCK cells is not likely due to a marked rate of passive diffusion that would obscure a mediated process since accelerated rates of diffusion were observed for cimetidine and daunomycin when the flux in MDCK cells was comparable to that of dTUB (Figure 2; legends of Figures 3 and 4).

Our interest in the renal transport of nucleosides initiated from measurements of Ado and dAdo in the body fluids of children lacking adenosine deaminase[20] and in adults treated with the deaminase inhibitor, deoxycoformycin[1]. Specifically, we found Ado to undergo net renal reabsorption, whereas dAdo exhibited a net renal secretion in humans and in mice. These were among the first reports of energy-requiring transport of these endogenous nucleosides. While others have focused on the mechanism for adenosine reabsorption, we have used dTUB to study the mechanism for dAdo renal secretion. Since mammalian kidney is capable of secreting organic cations and anions of diverse chemical structure by at least two, apparently independent, mechanisms (discussed in the Introduction), we tested the possible roles of these classical carriers in the renal secretion of dTUB. Not surprisingly, we found dTUB to be a substrate for the OCT by the known in vitro[3] and in vivo[2] criteria. Since neither of these carriers (i.e., organic cation or anion) have been purified, we focused attention on the possible role of Pgp in OCT. The time-dependent acquisition of cimetidine-sensitive uptake of TEA and NMN in kidney slices of newborn, developing mice failed to correlate with the developmental expression of Pgp protein and its mRNA[21]. Further, cimetidine and cyanine 863 (inhibitors of OCT) failed to reverse MDR in several tumor cells in culture, suggesting that Pgp is not responsible for OCT. In contrast to these findings, we recently discovered that cimetidine transepithelial flux is enhanced in the distal tubular cell line that has been transfected with human MDR1 cDNA (MDR-MDCK cells), indicating that Pgp is potentially capable of OCT, at least in cells that overexpress Pgp[22]. The observation that MDCK and MDR-MDCK cells do not transport dTUB (Table 1) is important, therefore, since dTUB may serve as a selective substrate for another putative OCT, as described below.

In many cells, the transmembrane movement of purine and pyrimidine nucleosides is mediated by facilitated diffusion systems that are or are not inhibited by p-nitrobenzylthioinosine[23]. Additionally, recent reports have demonstrated concentrative nucleoside transport that is coupled with the inwardly directed sodium gradient, i.e., synport systems[24]. With regard to Pgp, there have been few reports that implicate this MDR carrier to be associated with nucleoside transport, notable exceptions being azidothymidine and dideoxycytidine[16,17]. There is, therefore, reason to suspect that transport systems other than Pgp account for the transepithelial movement of many nucleosides. Other reasons to assume Pgp is not the only carrier for OCT are that OCT occurs by a "proton exchange" mechanism[25] whereas Pgp is an ATP-binding protein that possibly requires ATP hydrolysis for the energy-dependent transport[4]. The proton exchange mechanism would indirectly be driven by

the sodium-proton antiport and the basolaterally-located Na-K ATPase of renal proximal tubule cells, as illustrated below.

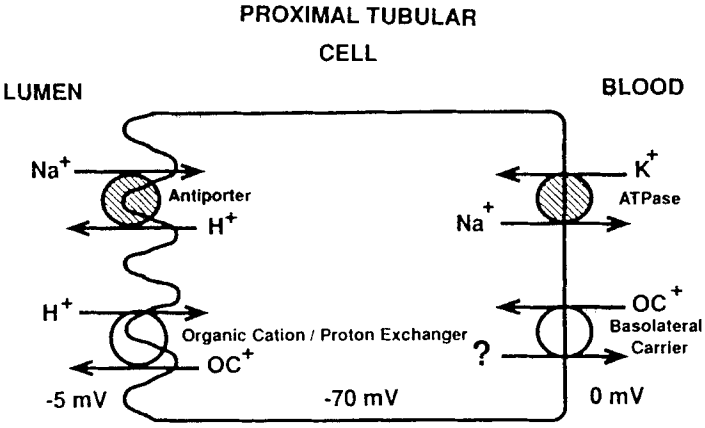

FIG. 6. A model for renal organic cation secretion. OC = organic cation. Adapted from Kinsella, et al.[6]

Although renal brush border membrane vesicles contain very little Pgp[21], proton-coupled exchange of TEA is readily demonstrable, whereas we have not observed proton-coupled exchange of this substrate or others in tumor cell membrane vesicles enriched with Pgp (unpublished observations). In summary, dTUB and a number of related nucleosides fail to inhibit Pgp-mediated transport of cimetidine and daunomycin in MDR-MDCK cells, and cross-resistance to Ado, dAdo and tubercidin is not observed in MDR tumor cells. These findings, coupled with previous studies of nucleoside and Pgp transport systems, suggest that dTUB (and perhaps other nucleosides) are transported by a system(s) other than Pgp, thereby making dTUB a relatively selective substrate to identify another OCT in mammalian kidney.

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