Epithelial Transport of Anthelmintic Ivermectin in a Novel Model of Isolated Proximal Kidney Tubules

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Purpose. The mechanism of excretion of the anthelmintic drug ivermectin was investigated in a novel experimental model of functionally intact proximal tubules isolated from a teleost fish (Fundulus heteroclitus).

Methods. Secretion into the lumens of freshly isolated proximal tubules was studied by means of confocal laser scanning microscopy and digital image analysis using ivermectin and fluorescent labelled ivermectin (BODIPY-ivermectin; BI) as substrates.

Results. The tubular cells rapidly accumulated BI from the medium and attained steady state within 25 minutes. Luminal fluorescence in the steady state was 5–7 times higher as compared to intracellular fluorescence. The secretion of BI into the tubular lumens was inhibited in a dose dependent manner by unlabelled ivermectin and inhibitors of the renal excretory membrane pump p-glycoprotein, namely SDZ PSC-833 and verapamil, but not by leukotriene C_4, a substrate of the renal export protein mrp2. Accumulation inside the tubular cells was not affected by the added inhibitors. Ivermectin inhibited the renal secretion of the fluorescent cyclosporin derivative NBDL-CS, a substrate of p-glycoprotein, but not the secretion of the mrp2-substrate fluorescein-methotrexate, nor the secretion of fluorescein, a substrate of the classical renal organic anion transporter.

Conclusions. The data are consistent with BI and ivermectin interacting in teleost kidney tubules exclusively with p-glycoprotein, but not with one of the other known excretory transport systems. In addition, the studies demonstrate that freshly isolated functionally intact kidney tubules from killifish are a useful tool to differentiate the substrate specificity of renal transport systems with respect to drug elimination.

KEY WORDS: ivermectin; p-glycoprotein; kidney; renal secretion; killifish.

INTRODUCTION

The ATP-binding cassette (ABC) transporters comprise a growing family of membrane-bound proteins that mediate several cell functions, including drug and toxicant export and ion homeostasis. Included in this family are several ATP-driven pumps, p-glycoprotein and the family of multidrug resistance associated transporters (mrps), that play major roles in determining drug uptake, distribution and excretion. They were originally discovered as overexpressed proteins in tumor cells with a multidrug resistant phenotype. Subsequently, p-glycoprotein and mrp2 were also found at high levels in certain excretory or barrier tissues, e.g., liver, renal proximal tubule, gut and brain capillary endothelium, where their polar distribution within the plasma membrane puts them in the correct orientation to 1) to drive xenobiotic excretion into bile and urine, and 2) to restrain the uptake of xenobiotics into blood and into protected fluid compartments, e.g., out of the gut or into the CSF. Along with drug metabolizing enzymes, such as the cytochrome P450-dependent enzymes, these transporters are important determinants of drug effectiveness on the one hand and of drug toxicity on the other hand. Pgp and mrp2 have similar distributions in epithelial tissues. In general, Pgp interacts with a wide range of uncharged and cationic drugs and mrp2 predominantly recognizes anionic compounds (1–5). However, there is some overlap in their substrate specificities, especially with respect to uncharged drugs. Thus, it is important to identify the carrier proteins responsible in tissues with multiple xenobiotic transporters.

Ivermectin (22,23-dihydroavermectin B_{1a}), a polycyclic lactone, is a semisynthetic analog of avermectin B1, which comes from *Streptomyces avermitilis*. It is used extensively to control and treat a broad spectrum of parasitic infections in human and veterinary medicine (6–8). Clinical safety and efficacy of ivermectin have been proven, e.g., in the treatment of blinding onchocerciasis (river blindness), which is caused by Onchocerca volvulus transmitted by the Simulium damnosum complex. However, some side effects, such as symptoms of central nervous dysfunction have been reported and ivermectin is contra-indicated in conditions associated with an impaired blood brain barrier, e.g., meningitis. In addition, after treatment of animals with drugs, which are p-glycoprotein (Pgp, mdr1-gene product) substrates, CNS side effects have been observed (9,10). This makes it likely, that ivermectin is interacting with this ATP-driven membrane located drug efflux pump. Since ivermectin exerts its side effects especially in the presence of Pgp substrates (11–14), it is of particular interest to know, by which mechanisms it is transported itself across epithelial barriers. Subsequently, attention should be paid on monitoring its impact on both the target (e.g., S. damnosum) and on animals (e.g., fish, crustaceans) that may be successors of ivermectin treated animals in the nutrition chain and therefore incorporate the drug nonvoluntarily to a high degree.

In the present study, we used renal proximal tubules from a teleost fish (killifish, *Fundulus heteroclitus*) and confocal microscopy to study 1) the excretory transport of a fluorescent ivermectin derivative (BODIPY-ivermectin, BI), and 2) the ability of ivermectin to affect the transport of fluorescent drugs handled by p-glycoprotein, mrp2 and the renal organic anion transport system. Renal tissue from teleost fish offers several important advantages for the study of excretory transport mechanisms in an intact, functioning epithelium (15,16). Killifish renal tubules are easily isolated and remain viable for hours to days. The killifish nephron consists almost exclusively of proximal tubule, the segment in which the drug transport

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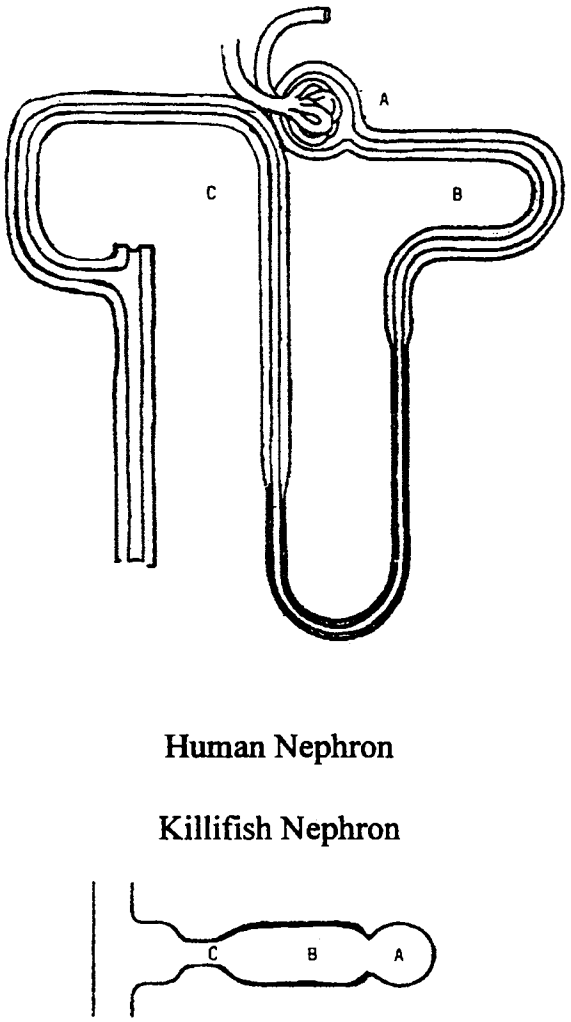

Fig. 1. Comparison of mammalian nephron and nephron from killifish (Fundulus heteroclitus). The renal tubule of the fish (adapted from 27) contains almost exclusively proximal segments but no distal parts. A = glomerulus, B = proximal tubule, C = distal tubule.

systems of interest are located (Fig. 1). During isolation, the broken ends of the tubules seal forming a closed, fluid-filled luminal compartment that communicates with the medium only through the tubular epithelium. Thus, the preparation has the correct geometry to facilitate the study of excretory (bath to urinary space) transport. A large body of evidence indicates that the xenobiotic transport mechanisms found in teleost tubules are functionally identical to those found in mammalian renal proximal tubules. Finally, the use of fluorescent substrates and confocal laser scanning microscopy allows the investigation of mechanisms driving transport across the luminal membrane in the intact tubule. In this regard, our recent studies have identified fluorescent substrates and specific inhibitors of p-glycoprotein, mrp2 and the classical renal organic anion transport system (17–19).

Here, we report that ivermectin blocks BI transport and that BI is transported by Pgp, but not mrp2 or the renal organic anion transport system. In addition, we demonstrate, that proximal tubules isolated from teleost fish are a useful and easy to handle tool to identify the molecular participants involved in drug transport.

MATERIALS AND METHODS

Chemicals

BI and fluorescein-methotrexate (FL-MTX) were purchased from Molecular Probes (Eugene, OR, USA). The fluorescent cyclosporin A derivative NBDL-CS was synthesized as described (17). The p-glycoprotein blocker SDZ PSC-833 was obtained from Novartis AG, Basle, Switzerland. All other chemicals were obtained from commercial sources at the highest purity available.

Animals and Tissue Preparation

Killifish (*Fundulus heteroclitus*) were purchased from local fishermen in the vicinity of Mount Desert Island, Maine, and maintained at the Mount Desert Island Biological Laboratory in tanks with natural flowing, aerated sea water.

Isolated renal tubules were prepared in marine teleost saline (20), containing in mM 140 NaCl, 2.5 KCl, 1.5 $CaCl_2$, 1.0 $MgCl_2$ and 20 Tris(hydroxy-methyl)-aminomethane (TRIS), pH 8.0. Renal tissue was teased under a dissecting microscope with fine forceps in order to remove adherent hematopoietic tissue. Individual killifish proximal tubules were dissected and transferred to a aluminum foil-covered, teflon incubation chamber containing 1.5 ml of marine teleost saline with fluroescent compound and added effectors. The chamber floor was a 4 × 4 cm glascover slip to which the tubules adhered lightly and through which the tissue could be viewed by means of an inverted confocal laser scanning microscope. The fluorescent compounds were dissolved in dimethylsulfoxide (DMSO) and added to the incubation medium. Preliminary experiments showed that the concentrations of DMSO used (<1%) had no significant effects on the uptake and distribution of the fluorescent labelled test compounds as measured by confocal and epifluorescence microscopy. Analysis by HPTLC of BI, FL-MTX and NBDL-CS after extraction with ethanol out of the tissue showed that the fluorescent compounds remained stable in the tissue for at least one hour.

Fluorescence Microscopy

The chamber containing tubules was mounted on the stage of a Olympus Fluo2 inverted confocal laser scanning microscope and viewed through a 40 × water immersion objective (NA = 1.15). The 488-nm laser line, a 510-nm dichroic filter and a 515-nm long-pass emission filter were employed. Low laser intensity (6% of maximum) was used to avoid photobleaching of the dyes. With the photomultiplier gain set to give an average luminal fluorescence intensity of 1500–3000 (full scale, 4096), tissue autofluorescence was undetectable. Using image capture and analysis software (NIH Image 1.61) incoming images could be displayed at the video rate on a high resolution computer monitor, and frame averages stored on a Jaz Drive for later analysis. To make a measurement, tubules in the chamber were first viewed under reduced, transmitted light illumination. A suitable field with several tubules was selected and an epi-fluorescence image was acquired by averaging 4 frames. It has been shown with a similar video microscopy system and glass capillary tubes filled with solutions of known concentrations of fluorescein that the relationship between image fluorescence intensity and fluorescein concentration is

approximately linear (21 and Miller, unpublished data). However, because there are uncertainties in relating cellular fluorescence to the actual concentration of an accumulated compound in cells with complex geometry (21,22), data are reported here as an average measured pixel intensity rather than an estimated concentration of the fluorescent labelled compounds.

Fluorescence intensities were measured from stored images using the Image 1.61 software as described previously (23). From each tubule under investigation, several adjacent cellular and luminal areas (100–300 pixels each) were selected. The background fluorescence intensity was substracted and then, the average pixel intensity for each area was calculated. The values used for that tubule were the means for all selected areas.

Statistics

Data are given as mean ± SE. Means were considered to be statistically different, when the probability value (P) was less than 0.05 by use of an unpaired t-test.

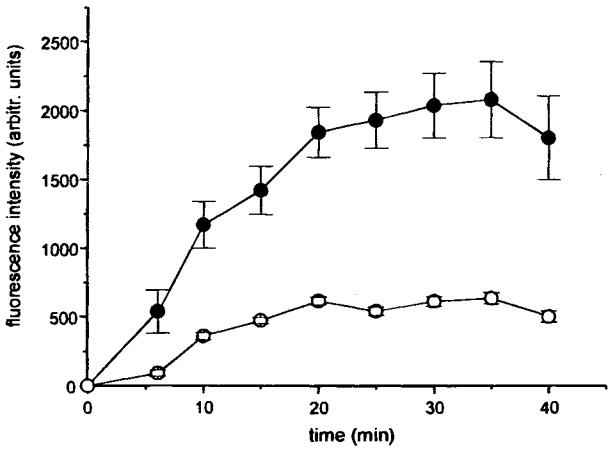

Fig. 3. Time course of transport of BODIPY-Ivermectin in killifish proximal tubules. Tubular tissue was incubated with 1 M BODIPY-ivermectin in teleost Ringer solution (means ± SE of n = 12).

RESULTS

When killifish renal proximal tubules were incubated in media with micromolar concentrations of BI, the fluorescent compound was seen to accumulate in the tissue. Figure 2 shows a typical confocal image of a tubule after 30 min incubation in medium with 1 μM BI. The image shows that the tubular epithelium clearly accumulated the compound, but that significantly higher levels were present in the lumen. The fluorescence intensity in the incubation medium was substantially lower than that of the tissue. This is the same overall pattern of fluorescence seen in micrographs of killifish tubules exposed to dyes handled by specific renal excretory transport systems present in this tissue and has been taken to indicate secretion into the urinary space (17–19,23).

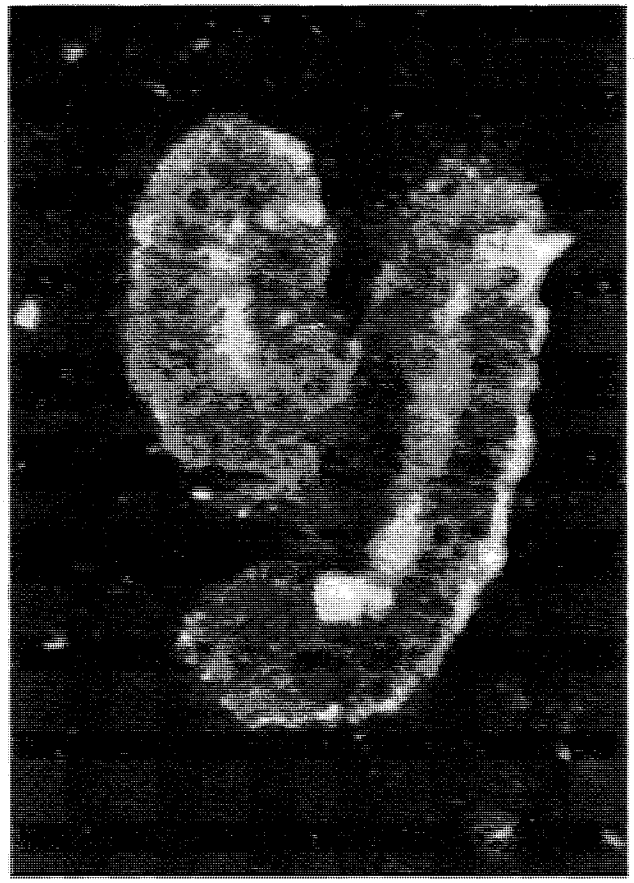

Fig. 2. Confocal micrograph of killifish tubules incubated in medium with 1 μM BODIPY-ivermectin.

Figure 3 shows the time course of accumulation of fluorescence in the cells and lumens of killifish proximal tubules incubated in medium with 1 μM BI. In both tissue compartments, fluorescence increased rapidly over the first 20–30 min and then reached a plateau. At all time points, luminal fluorescence significantly exceeded cellular fluorescence; at steady state (30–45 min) the lumen to cell fluorescence ratio was 5–7. Separate experiments showed that 1 mM NaCN significantly reduced steady state luminal fluorescence, but had no effect on cellular fluorescence (luminal and cellular fluorescence in control tubules averaged 2432 ± 192 and 656 ± 96 fluorescence units, respectively; corresponding values in NaCN-exposed tubules were 480 ± 128 and 560 ± 32 units). These data from confocal images indicate that BI accumulates in the cells and lumens of proximal tubules, but that only luminal accumulation is dependent on cellular metabolism.

When the tubules were incubated with 1 μM BI and increasing concentrations of ivermectin, luminal, but not cellular, fluorescence decreased and these decreases were dependent on the concentration of ivermectin (Fig. 4). Since the CNS-related side effects of ivermectin may occur in the presence of p-glycoprotein inhibitors or in p-glycoprotein knock out animals (9–12), we determined the effects of p-glycoprotein substrates and inhibitors on BI transport. Figure 5 shows that BI transport into the tubular lumen was significantly decreased in the presence of 10 μM SDZ PSC-833 and verapamil; neither compound affected cellular fluorescence. Figure 5 also shows that LTC_4, a potent inhibitor of transport mediated by mrps, but not p-glycoprotein, had no effect on BI transport.

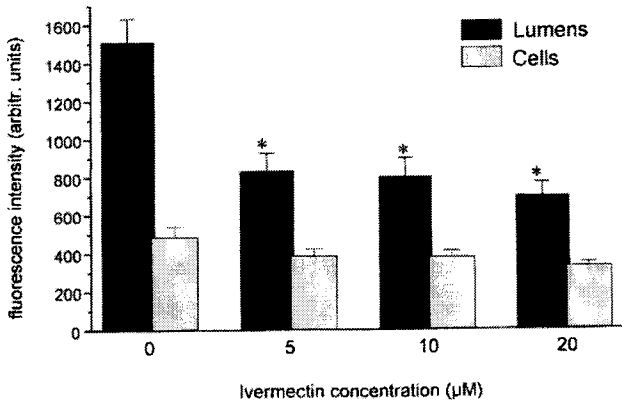

Fig. 4. Effects of ivermectin on the transport of BODIPY-ivermectin. Tubules were incubated in medium with 1 μM BODIPY-ivermectin without or with the indicated concentration of ivermectin. Data are given as mean ± SE for 10 tubules.

These data indicate that the fluorescent ivermectin derivative is a substrate for transport by p-glycoprotein in killifish renal tubules. To determine which transporters ivermectin itself interacts with, we measured the effects of ivermectin on the tubular secretion of three fluorescent drugs, each of which is actively transported by a different system in killifish tubules. 1) NDBL-CS enters proximal tubule cells by simple diffusion and is secreted into the lumen by p-glycoprotein (17). In agreement with previous studies, cell to lumen transport of NBDL-CS was reduced by verapamil and SDZ PSC-833, but not by LTC_4 (Fig. 6A) or probenecid (17). 2) Fl-MTX enters proximal tubule cells on a Na-independent transporter for large organic anions and is secreted into the lumen on Mrp2 (18). FL-MTX transport from cell to lumen was reduced by LTC_4, but not by verapamil (Fig. 6B). 3) FL enters on cells on the Na-dependent transporter for small organic anions and is secreted into the lumen by an as yet unidentified carrier. In agreement with previous studies cellular and luminal accumulation of FL was

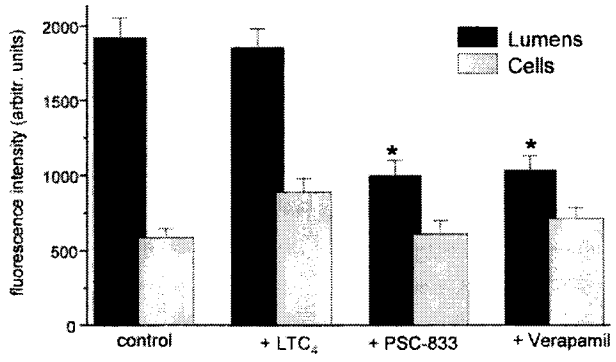

Fig. 5. Effects of inhibitors of p-glycoprotein and Mrp2 on BODIPY-ivermectin transport. Tubules were incubated in medium with 1 μM BODIPY-ivermectin without (control) or with 10 μM SDZ PSC-833 or verapamil or 0.5 μM LTC_4. Data are given as mean ± SE for 12 tubules (*: significantly lower than controls, $P < 0.05$).

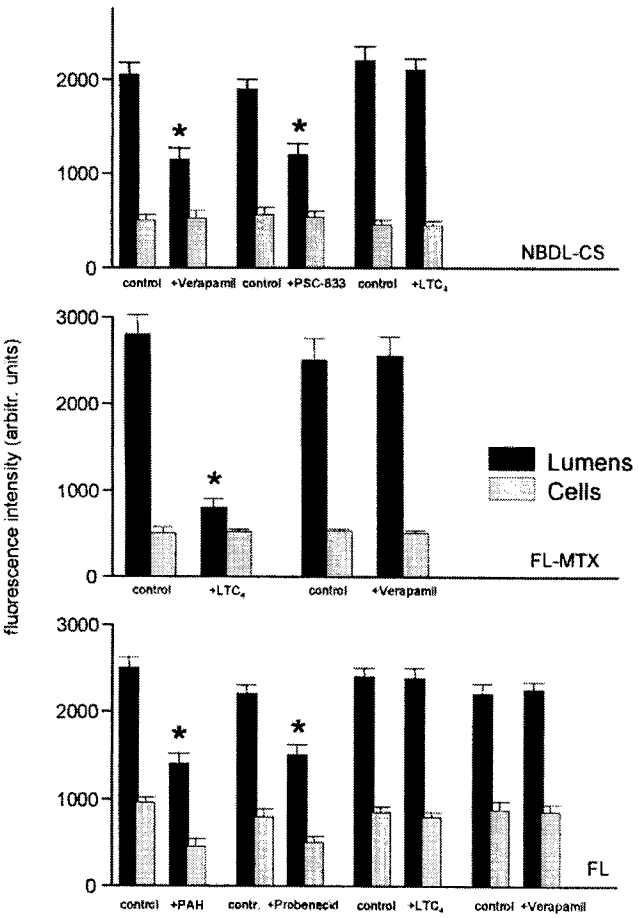

Fig. 6. Transport of NBDL-CS, FL-MTX and FL in killifish renal proximal tubules. Tubules were incubated in medium with 1 μM fluorescent compound without (control) or with 10 μM SDZ PSC-833 or verapamil, 0.5 μM LTC_4 or 100 μM P-aminohippurate (PAH). Data are given as mean ± SE for 7 tubules (*: significantly lower than controls, $P < 0.05$).

reduced by PAH and probenecid, but not by LTC_4 or verapamil (Fig. 6C).

Figure 7 shows that ivermectin only significantly reduced transport for one of the three substrates. It did not affect transport of FL-MTX or FL, but, like verapamil and SDZ PSC-833, ivermectin reduced the cell to lumen transport of NBDL-CS. Luminal accumulation of NBDL-CS fell with increasing ivermectin concentration.

DISCUSSION

The results of the present study show that a fluorescent ivermectin derivative (BI) was transported from bath to urinary space of killifish renal proximal tubules by a process that was concentrative, specific and energy-dependent. BI transport from bath to epithelial cell was not affected by NaCN nor by any other compound tested, suggesting that this step in transepithelial transport was not carrier mediated. In contrast, transport from cell to tubular lumen was reduced by NaCN, verapamil, SDZ PSC 833 and by ivermectin itself, but not by LTC_4.

This pattern of effects is consistent with diffussive BI uptake at the basolateral membrane of the epithelial cells followed by carrier mediated and energy-dependent efflux across

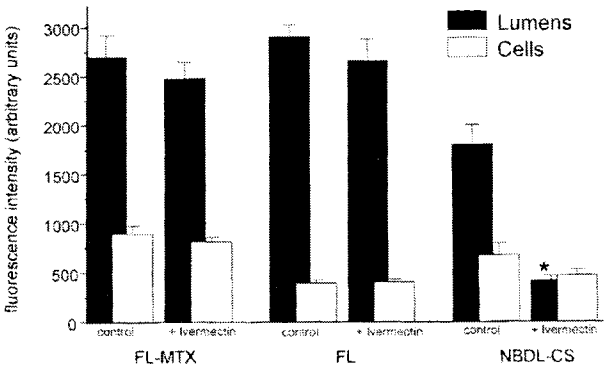

Fig. 7. Effects of ivermectin on transport of NBDL-CS, FL-MTX and FL. Tubules were incubated in medium with 1 μM fluorescent compound without (control) or with the indicated concentration of ivermectin. Data are given as mean ± SE for 12 tubules (*: significantly lower than controls, $P < 0.05$).

the luminal membrane. The fact, that no total inhibition of secretion was observed, can be explained by a diffusional component in the excretory step. Based on the known specificities of the compounds that inhibit luminal transport of Bl, excretion appears to be mediated by p-glycoprotein. We have previously shown similar transport patterns for fluorescent derivatives of CSA and rapamycin (17,19). For both of these compounds, we found no significant effect of luminal transport inhibitors on steady state cellular accumulation; this was taken to mean that factors other than luminal efflux were the major determinants of cellular accumulation. Consistent with the transport experiments using Bl as substrate, when ivermectin was tested as an inhibitor, it reduced cell to lumen transport of NBDL-CS, a process that is mediated by p-glycoprotein (17). In contrast, transport of FL-MTX and of FL was not affected. These are substrates for the Na-independent and Na-dependent renal organic anion transport systems, respectively. The luminal step of FL-MTX transport is mediated by the ABC transporter, Mrp2. Both transport systems are particularly sensitive to reductions in cell metabolism and the lack of effect of ivermectin on these transport processes can be taken as evidence that the drug did not affect transport of NBDL-CS through metabolic inhibition. In addition, preliminary experiments showed that 1 mM TEA (tetraethylammonium) did not influence Bl-secretion into the tubular space (data not shown), suggesting that an organic cation transporter is not involved.

Together, the present results suggest that both Bl and ivermectin are substrates for transport by p-glycoprotein in renal proximal tubule. This is the first evidence for transport of this drug via a distinct transport system in the kidney and the first data showing excretory transport of the drug across an intact, native epithelium. In animals, ivermectin is mainly recovered in faeces and the highest drug concentrations are found in liver and fat. The long terminal half-life of about 35 hours (24) and the apparent volume of distribution of more than 47 L (25) are indicative for a high protein binding and therefore, renal excretion could be masked by binding and reabsorption in the distal part of the tubules, similar as it has been observed for other lipophilic drugs, such cyclosporin A (17).

The mechanism by which ivermectin is transported is of special interest, since it is a widely used anthelmintic in veterenary medicine with a well described side effect profile. One of the best known side effects of ivermectin may be episodes of neurotoxicity (3,4). Published data suggest, that this neurotoxicity may be caused by a blockade of p-glycoprotein by ivermectin. It has also been found, that resistance to ivermectin becomes an augmenting problem in parasite control. A Pgp-like protein is thought to play a role in ivermectin resistance in the sheep nematode parasite Haemonchus contortus (26). Expression of p-glycoprotein mRNA was higher in ivermectin-selected than unselected strains of H. contortus. In addition, the multidrug resistance reversing agent verapamil increased the efficacy of ivermectin against a selected strain of this nematode. Thus, it is conceivable, that ivermectin directly interacts with a p-glycoprotein in the parasite.

Finally, the present results, along with our recent reports (17–19), show the killifish renal proximal tubule to be a powerful model for the study of drug excretory transport mechanisms in an intact, functioning native epithelium. As mentioned in the introdution, the model has the advantages of simple proximal tubule preparation and extended viability. Using fluorescent substrates and confocal microscopy, we have been able to visualize each step in transport for all the major systems in proximal tubule. Several of the transporters involved, e.g., p-glycoprotein and mrp2, are present in other epithelia (liver and intestine) and in other barrier tissues (brain capillary epithelium) as well as in proximal tubule.

To date, the killifish model has proven its usefullness in studies focussed on 1) mechanisms of transport of drugs and of fluorescent drug derivatives. 2) interactions between drugs and specific transporters.

ACKNOWLEDGMENTS

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