p-Glycoprotein-Mediated Transport of a Fluorescent Rapamycin Derivative in Renal Proximal Tubule

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Accepted for publication March 5, 1997

ABSTRACT

The transport of a fluorescent rapamycin derivative was measured in killifish (Fundulus heteroclitus) renal proximal tubules by means of confocal microscopy and image analysis. Renal cells and tubular lumens rapidly accumulated the rapamycin analog from the medium and attained steady state within 60 min. At steady state, luminal fluorescence intensity was two to four times higher than cellular fluorescence. Cellular fluorescence intensity was a linear function of medium substrate concentration and was not affected by any treatment used. In contrast, luminal fluorescence exhibited a saturable component as the medium concentration of the rapamycin derivative was increased. Secretion into the lumen was blocked by KCN, rapamycin, cyclosporin A and substrates for p-glycoprotein (verapamil, PSC-833 and FK506), but not by substrates for the renal organic anion or organic cation transport systems, such as p-aminohippurate, leukotriene C₄ or tetraethylammonium. Finally, rapamycin blocked p-glycoprotein-mediated secretion of a fluorescent cyclosporin A derivative. The data are consistent with the fluorescent rapamycin analog entering proximal tubule cells by simple diffusion and then being pumped into the tubular lumen by p-glycoprotein. They suggest that the parent compound, rapamycin, would be handled similarly.

Rapamycin, is a potent, macrocyclic immunosuppressive that blocks transduction of cytokine signals needed for proliferation and maturation of T cells (Sehgal and Bansbach., 1993; Sehgal et al., 1994; Kahan et al., 1995; Dumont and Su, 1996). The therapeutic potency of the drug is up to 100 times greater than that of CSA with a relatively long half-life in animals and humans (Yatscoff et al., 1992; Yatscoff, 1996). In addition, there is evidence that the renal side effects of rapamycin, such as tubular atrophy and interstitial fibrosis, are less severe than for CSA (Thiliveris et al., 1995). At present, it is not clear whether specific drug transport systems handle rapamycin or whether it distributes through the body by simple diffusion. Available data suggest that rapamycin may be capable of functioning as a MDR-reversing agent by interacting with p-glycoprotein, thereby increasing the cytotoxicity of chemotherapeutic agents in multidrug-resistant cells (Arceci et al., 1992). The increase in cytotoxic drug accumulation was observed at concentrations of rapamycin that were similar to those shown to be effective for other MDR reversal agents, such as CSA and verapamil.

These findings document an interaction between rapamycin and p-glycoprotein, but they do not address the question of whether the drug is a substrate for transport. In the present study we used confocal microscopy and digital image analysis to study the transport of a fluorescent rapamycin derivative, NBD-rapamycin (fig. 1), in intact renal proximal tubules from a teleost fish. Renal tissue from teleost fish offers several important advantages for the study of secretory transport mechanisms (Pritchard and Miller, 1991). Teleost kidneys contain a high proportion of proximal tubules that are easily isolated and remain viable for long periods. When tubules are isolated, broken ends rapidly reseal to form a closed, fluid-filled luminal compartment that only communicates with the medium through the tubular epithelium. Thus, this tissue has the appropriate geometry for the study of secretion in intact tubules. When teleost tubules are used along with fluorescent substrates and quantitative fluorescence microscopy, the mechanisms driving both uptake by the cells and secretion into the tubular lumen can be investigated (Miller and Pritchard, 1991; Miller, 1995; Schramm et al., 1995). Finally, secretory transport mechanisms found

Received for publication November 22, 1996.

1 Supported in part by a grant from the Deutsche Forschungsgemeinschaft to G.F. and a travel grant from Mundipharma Pharmaceuticals to J.D. This study was also supported by a NATO CRG grant.

ABBREVIATIONS: CSA, cyclosporin A; DMSO, dimethylsulfoxide; LTC₄, leukotriene C₄; MDR, multidrug resistance; NA, numerical aperture; NBD-rapamycin, (4-nitrobenzofurazan-7-yl)-rapamycin; NBLD-CSA, [N-(4-nitrobenzofurazan-7-yl)-o-Lys8]-cyclosporin; PAH, p-aminohippurate; TEA, tetraethylammonium.
in teleost tubules appear to be identical with those found in mammalian proximal tubule (Pritchard and Miller, 1991, 1993) and among these is a potent p-glycoprotein-mediated drug transport pathway (Miller, 1995; Schramm et al., 1995). The results of the present study show that NBD-rapamycin entered killifish proximal tubule cells by simple diffusion and was pumped from cell to tubular lumen by p-glycoprotein.

Materials and Methods

Chemicals. Rapamycin was obtained from Sigma (St. Louis, MO). The fluorescent rapamycin derivative was synthesized by coupling the fluorescent residue 7-bromo-4-nitrobenzofurazan (Merck, Darmstadt, Germany) to rapamycin. NBD-rapamycin was purified from the reaction mixture by high-performance liquid chromatography. NBDL-CSA was synthesized as described previously (Schramm et al., 1995). All other chemicals were obtained from commercial sources at the highest purity available.

Animals and tissue preparation. Killifish (Fundulus heteroclitus) were collected by local fishermen in the vicinity of Mount Desert Island, Maine and maintained in tanks with natural, flowing sea water at the Mount Desert Island Biological Laboratory. For some experiments, killifish were collected near Duke University Marine Laboratory (Beaufort, NC) and maintained in tanks with recirculating, artificial sea water at the National Institute of Environmental Health Sciences.

Renal tubular masses were isolated in a marine teleost saline based on that of Forster and Taggart (1950), containing (in mM): NaCl, 140; KCl, 2.5; CaCl₂, 1.5; MgCl₂, 1.0; and tris(hydroxymethyl)aminomethane, 20; at pH 8.0. All experiments were carried out at 18–20°C. Under a dissecting microscope each mass was teased with fine forceps to remove adherent hematopoietic tissue. For microscopy, individual killifish proximal tubules were dissected free of the masses and transferred to a foil-covered Teflon chamber (Bionique) containing 1 ml of marine teleost saline with 1 to 5 μM fluorescent compound or precursor. The chamber floor was a 4 × 4 cm glass coverslip to which the tubules adhered lightly and through which the tissue could be viewed by means of an inverted microscope (below).

NBD-rapamycin was dissolved in DMSO and added to the incubation medium. Previous studies have shown that the concentrations of DMSO used here (0.05–1%) had no significant effects on the transport of fluorescein, NBDL-CSA and daunomycin (Schramm et al., 1995; Miller, 1995). Initial experiments demonstrated that transport of NBD-rapamycin was not altered by DMSO in the indicated concentration range.

The ability of the tissue to degrade NBD-rapamycin was tested by high-performance thin-layer chromatography with fluorescence detection. Pooled tubules from six fish were incubated for 1 hr with 1 μM fluorescent compound and then extracted with chloroform/ethanol (2:1 vol/vol). The extract was centrifuged and the supernatant was evaporated. The residue was dissolved in 100 μl chloroform/ethanol and subjected to high-performance thin-layer chromatography. Fluorescence detection revealed no degradation of NBD-rapamycin.

Confocal fluorescence microscopy. Tubules in the chamber were mounted on the stage of a confocal microscope. Two systems were used for the present studies. Most of the images were collected with a Nikon 40 × Fluor oil immersion objective (NA, 1.4). Illumination was provided by an Ar laser at 488 nm. A 510-nm dichroic filter and a 515-nm long-pass emission filter were used. Some images were collected by a Zeiss model 410 Inverted Laser Scanning Confocal Microscope with a Zeiss 40 × plan-neofluar water immersion objective (NA, 1.3). The microscope was fitted with an Ar-Kr laser also providing light at 488 nm. A 510-nm dichroic filter and a 515-nm long-pass emission filter were used. Neutral density filters and reduced laser power were used to minimize photobleaching. Preliminary experiments showed that under these conditions fluorescence intensities in cells and tubular lumens were reduced by less than 5% from one collected image to the next (below). With these settings, and with photomultiplier gain adjusted so that the average pixel intensity in the lumens of control tubules was 50–150, tissue autofluorescence was undetectable.

To obtain an image, dye-loaded tubules in the chamber were viewed under reduced, transmitted light illumination, and a single proximal tubule with well-defined lumen and undamaged epithelium was selected. The plane of focus was adjusted to cut through the center of the tubular lumen. Then, in confocal fluorescence mode, 128 video frames were averaged (Noran) or a single 8-sec scan of the tubule was collected (Zeiss). The confocal image (512 × 512 × 8 bits) was viewed on a high-resolution monitor and saved to an optical disk. Fluorescence intensities were measured from stored images by an Apple Power Macintosh 7100 computer and NIH Image version 1.58 software as described previously (Miller, 1995; Masereeuw et al., 1996). Three to five adjacent cellular and luminal areas (at least 200–400 pixels each) were selected from each tubule. After background subtraction, the average pixel intensity for each area was calculated. Then the lumen-to-cell fluorescence ratio for each pair of adjacent areas was calculated. The values used for that tube were the means of all measured areas.

Statistics. Data are given as means ± S.E. Means were considered to be statistically different, when the probability value (P) was less than .05 by use of the appropriate paired or unpaired t test.

Results

In the present experiments, killifish renal proximal tubules were incubated in medium containing a fluorescent rapamycin derivative, confocal fluorescence images were collected and fluorescence distribution patterns analyzed to obtain an indication of dye distribution in the tissue. Data are presented as steady-state fluorescence intensity measurements made over the cellular and luminal regions of the...
tubules. Two caveats must be kept in mind when interpreting such measurements. First, the signal from a fluorescent probe is sensitive to environment, e.g., pH or solvent polarity. As a result, absolute calibration of dye concentration in a single region of a tissue or a cell is difficult and the constant relating probe fluorescence to concentration could vary from region to region. Second, the steady-state solute concentration in a tissue compartment is a function of all processes governing entry into and exit from that compartment. Changes in steady-state solute concentration indicate that one or more of those processes has been altered. Often, additional knowledge about the nature of the treatment causing the change in concentration can be used to eliminate possibilities.

When killifish renal proximal tubules were incubated in media with micromolar concentrations of NBD-rapamycin, the fluorescent compound was seen to accumulate in the tissue. Figure 2 shows a typical confocal image of a tubule after 60 min incubation in medium with 1 μM NBD-rapamycin. 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 indicates secretion into the urinary space (Schramm et al., 1995; Miller, 1995).

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 NBD-rapamycin. In both tissue compartments, fluorescence increased rapidly over the first 30 min and then reached a plateau after about 60 min. At all but the earliest time point, luminal fluorescence significantly exceeded cellular fluorescence; at steady state (60–90 min) the lumen-to-cell fluorescence ratio averaged 2.3. In experiments with 94 control tubules from eight fish this ratio averaged 3.3 (range, 1.8–4.1), which indicated some fish-to-fish variability. Figure 3 also shows that addition of 1 mM KCN to the medium had no effect on cellular fluorescence, but significantly reduced luminal fluorescence. In KCN-exposed tubules the lumen-to-cell fluorescence ratio was slightly less than unity. These data from confocal images indicate that NBD-rapamycin accumulates in the cells and lumens of proximal tubules, but that only luminal accumulation is dependent on cellular metabolism.

The steady-state accumulation and distribution of NBD-rapamycin was concentration dependent (fig. 4). Cellular fluorescence intensity increased linearly with increasing medium NBD-rapamycin concentration, but luminal fluorescence intensity appeared to saturate with higher concentrations. As a result, the lumen-to-cell fluorescence ratio decreased from 4 at 1 μM NBD-rapamycin to 1.8 at 10 μM NBD-rapamycin.

Table 1 shows the effects of various agents on the transport of 0.5 to 1.0 μM NBD-rapamycin by killifish tubules. None of the compounds tested altered cellular fluorescence, even though several greatly reduced luminal fluorescence. The parent compound, rapamycin, was a potent inhibitor of luminal accumulation of NBD-rapamycin, with 1 μM rapamycin reducing luminal fluorescence by more than 60% and 5 μM reducing luminal fluorescence by more than 80%. Other inhibitors of luminal accumulation included CSA, PSC-833, FK506 and verapamil (table 1). Some of these (PSC-833 and FK506) were effective at submicromolar concentrations. All of these drugs are substrates for or modifiers of p-glycoprotein. In contrast, inhibitors of the other xenobiotic transport systems present in killifish proximal tubule, PAH for the classical organic anion system (Miller and Pritchard, 1991), TEA for the organic cation system (Miller, 1995) and LTC4 for the Na-independent system for large organic anions (Masureeuw et al., 1996), used at concentrations that should have blocked transport on those systems, were without effect (table 1).

Finally, we tested the effects of the parent compound, rapamycin, on the transport of a known substrate for p-glycoprotein, NBDL-CSA, a fluorescent derivative of the immunosuppressive drug, CSA (Schramm et al., 1995). Figure 5 shows that rapamycin, at 1 and 5 μM, reduced luminal fluorescence in a concentration-dependent manner; rapamycin had no effect on cellular fluorescence. These data, together with the results showing that both rapamycin and CSA block secretion of NBD-rapamycin, are consistent with CSA and rapamycin and their NBD derivatives being handled by a common luminal transporter, p-glycoprotein.

**Discussion**

Specific membrane transport proteins, e.g., MDR transporters and MDR-associated proteins, are drug-transporting ATPases found in tumor cells, normal cells and also in excretory epithelia (kidney and liver). By mediating active drug efflux from cells and from the body they play a major role in determining drug concentrations reaching sensitive sites on cell surfaces and within cells. Along with drug-metabolizing enzymes, these transporters can be important determinants of drug effectiveness on the one hand and drug toxicity on the other hand. Moreover, because of their wide specificity limits, these transporters also provide a mechanism, competition for transport, by which drugs with very different structures may interact. Thus, it is important to understand not only interactions with enzymes that may metabolize the drugs and modify their actions, but also interactions with specific drug transporters that determine distribution and excretion patterns.
The present experiments demonstrate that a fluorescent analog of rapamycin, NBD-rapamycin, is taken up by killifish renal proximal tubules and is transported into the tubular lumen by a concentrative process. As with other secreted organic solutes, NBD-rapamycin transport from bath to urinary space was a two-step process, which consists of uptake at the basolateral membrane of the tubular epithelial cells followed by efflux from cell to lumen. However, very different mechanisms were responsible for the two steps in NBD-rapamycin secretion. Uptake of NBD-rapamycin through the basolateral plasma membrane was not affected by unlabeled rapamycin or by other inhibitors of membrane transport or cellular metabolism. Cellular uptake was a linear function of medium concentration of the fluorescent derivative. Thus, it is likely that NBD-rapamycin crossed the basolateral membrane of these renal cells by simple diffusion. In killifish tubules, a similar diffusive uptake step has been demonstrated in the secretory transport of two other p-glycoprotein substrates, a fluorescent derivative of CSA and daunomycin (Schramm et al., 1995; Miller, 1995). As with the fluorescent CSA derivative, the apparent accumulation of NBD-rapamycin within renal cells (present study) could be explained by invoking passive mechanisms, such as partitioning into lipid-rich intracellular compartments and binding to proteins (immunophillins).

In contrast to uptake across the basolateral membrane, transport across the luminal membrane had all the hallmarks of an active, carrier-mediated process. First, the level of NBD-rapamycin-derived fluorescence measured in the luminal compartment exceeded that in the cell. Because the luminal space is a simple aqueous compartment, it is unlikely that this increase in fluorescence is caused by compartmentation, e.g., binding to macromolecules or partitioning into lipid-rich regions. Second, NBD-rapamycin secretion was blocked when metabolism was inhibited by KCN. Third, transport from cell to lumen appeared to saturate as the medium NBD-rapamycin concentration was increased. Finally, NBD-rapamycin transport from cell to lumen was in-

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**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Fluorescence Intensity</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Cell</td>
</tr>
<tr>
<td>Control (0.5 µM)</td>
<td>12</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>1 µM rapamycin</td>
<td>7</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>5 µM rapamycin</td>
<td>9</td>
<td>34 ± 7</td>
</tr>
<tr>
<td>5 µM CSA</td>
<td>11</td>
<td>25 ± 4</td>
</tr>
<tr>
<td>Control (1.0 µM)</td>
<td>9</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>1 mM PAH</td>
<td>5</td>
<td>19 ± 3</td>
</tr>
<tr>
<td>1 mM TEA</td>
<td>7</td>
<td>17 ± 1</td>
</tr>
<tr>
<td>0.5 µM LTC4</td>
<td>6</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>100 µM Verapamil</td>
<td>7</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>Control (1.0 µM)</td>
<td>7</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>0.1 µM FK506</td>
<td>7</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>1.0 µM FK506</td>
<td>7</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>Control (1.0 µM)</td>
<td>8</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>0.1 µM PSC-833</td>
<td>9</td>
<td>13 ± 1</td>
</tr>
<tr>
<td>0.5 µM PSC-833</td>
<td>7</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>1.0 µM PSC-833</td>
<td>7</td>
<td>15 ± 2</td>
</tr>
</tbody>
</table>

*Significantly lower than controls, P < .05; **significantly lower than controls, P < .01.
Based on the lack of effect of PAH, TEA and LTC₄ on the luminal fluorescence, luminal fluorescence was significantly reduced bar shows the mean fluorescence intensity for six to eight tubules; 100 m was not a result of disruption of cellular energy metabolism. This inhibition was not a result of disruption of cellular energy metabolism. Incubating killifish tubules for 60 min with 5 μM CSA, 50 to 100 μM verapamil or 5 μM rapamycin did not affect the active secretion of fluorescein via the organic anion system (Schramm et al., 1995; Miller, 1995, unpublished data). If these compounds had affected cell metabolism we would have expected to see inhibition of fluorescein transport, because the organic anion system is particularly sensitive to agents that disrupt metabolism or reduce the transmembrane Na gradient (Pritchard and Miller, 1993). Thus, it appears that NBD-rapamycin shares a specific, luminal transport system with several other compounds, and among these is unlabeled rapamycin.

Renal proximal tubule has been shown to possess four distinct transport pathways for organic xenobiotics (Pritchard and Miller, 1993; Masereeuw et al., 1996; Schramm et al., 1995; Miller, 1995): 1) the classical organic anion system (model substrate, PAH); 2) the organic cation system (model substrate, TEA); 3) a Na-independent system for large organic anions (best inhibitor, LTC₄); and 4) p-glycoprotein. Based on the lack of effect of PAH, TEA and LTC₄ on the transport of NBD-rapamycin (table 1), it is unlikely that the first three pathways are involved in the transport from cell to lumen of this fluorescent derivative. Rather, all compounds that were effective inhibitors of NBD-rapamycin secretion were also substrates for or modifiers of p-glycoprotein. Consistent with NBD-rapamycin and unlabeled rapamycin being handled by p-glycoprotein in killifish tubules, rapamycin was a potent inhibitor of the secretion of NBDL-CSA, a substrate that is transported only by p-glycoprotein (Schramm et al., 1995).

The present study is the first to investigate mechanisms of rapamycin transport in any cell type. Assuming that rapamycin and its NBD derivative are handled similarly, the data suggest that in renal proximal tubule both compounds are substrates for transport by p-glycoprotein. Note that this drug-transporting ATPase is expressed in both tumors and normal tissues. As a result, one would expect rapamycin to compete for transport with the large number of drugs and endogenous compounds handled by that transporter. Such interactions at the transporter level could alter drug levels in target cells and in plasma and could also shift urine and bile drug excretion patterns.

Fig. 5. Inhibition of NBDL-CSA transport by rapamycin. Tubules were incubated in medium containing 1.0 μM NBDL-CSA without (control) or with 1 or 5 μM rapamycin. After 60 min, confocal images were acquired and then analyzed as described under “Materials and Methods.” Each bar shows the mean fluorescence intensity for six to eight tubules; variability is given by S.E. bars. Rapamycin had no significant effects on luminal fluorescence. Luminal fluorescence was significantly reduced by 1 μM (P < .05) and 5 μM (P < .01) rapamycin.

hhibited by micromolar concentrations of unlabeled rapamycin, CSA, PSC-833, FK506 and verapamil. This inhibition was not a result of disruption of cellular energy metabolism. Incubating killifish tubules for 60 min with 5 μM CSA, 50 to 100 μM verapamil or 5 μM rapamycin did not affect the active secretion of fluorescein via the organic anion system (Schramm et al., 1995; Miller, 1995, unpublished data). If these compounds had affected cell metabolism we would have expected to see inhibition of fluorescein transport, because the organic anion system is particularly sensitive to agents that disrupt metabolism or reduce the transmembrane Na gradient (Pritchard and Miller, 1993). Thus, it appears that NBD-rapamycin shares a specific, luminal transport system with several other compounds, and among these is unlabeled rapamycin.

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References


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