Fluorescein-methotrexate transport in rat choroid plexus analyzed using confocal microscopy

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Fig. 1. Distribution of transporters for anionic xenobiotics in rat choroid plexus epithelial cells. Only shown are those transporters that have been immunolocalized to a specific side of the cell (7, 11, 12, 13, 15, 24). In addition, high to moderate levels of mRNA for organic anion transporter (Oat) 2, multidrug resistance-associated protein (Mrp) 4, and Mrp5 were detected in the tissue; very low levels of mRNA for Oat1, Oatp1, Oatp4, Oatp5, Oatp12, Oat-K1/2, Mrp 2, and Mrp3 were also detected (5). At present, it is not clear to what extent any of these transport proteins are expressed in the tissue. CSF, cerebrospinal fluid.

the National Institute of Environmental Health Sciences Animal Use Committee. Tissue was immediately transferred to ice-cold, artificial cerebrospinal fluid (aCSF: 103 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 25 mM NaHCO3, 10 mM glucose, and 1 mM sodium pyruvate, at pH 7.4), previously gassed with 95% O2 -5% CO2. Preliminary experiments showed that the incubation medium was the apical plasma membrane. In Fig. 2, B and D, erythrocytes can be seen within the blood vessels as areas of very low fluorescence, indicating exclusion of FL and FL-MTX from these cells or quenching of fluorescence by Hb. In spite of the low fluorescence within erythrocytes, spaces between and around these cells are highly fluorescent, with the fluorescence intensity being roughly equivalent to that seen in the subepithelial space.

FL-MTX transport. Figure 2 shows transmitted light and confocal micrographs of choroid plexus tissue after 45 min incubation in aCSF containing 1 μM FL or 2 μM FL-MTX. In the transmitted light images, the apical brush-border membrane appears as a refractile element between cells and medium (Fig. 2, A and C). At high magnification, the epithelial cells are seen to contain a large central nucleus and smaller vesicular organelles (2). Beneath the epithelium is a distinct subepithelial space and blood vessels containing erythrocytes. Unlike most other capillaries in the brain, those in choroid plexus are fenestrated and thus the endothelium is no barrier to exchange of solutes between the vasculature and the interstitium. As shown previously (2), FL, accumulated in both the epithelial cells and the subepithelial/vascular spaces, with fluorescence intensities in vascular/subepithelial spaces > cells > medium (Fig. 2B). The pattern of FL-MTX fluorescence was clearly different, with levels in the cells slightly lower than the medium and levels in vascular/subepithelial spaces substantially higher than the medium (Fig. 2D). The only cellular element exhibiting FL-MTX fluorescence higher than the medium was the apical plasma membrane. In Fig. 2, B and D, erythrocytes can be seen within the blood vessels as areas of very low fluorescence, indicating exclusion of FL and FL-MTX from these cells or quenching of fluorescence by Hb.

To acquire images, the chamber containing tissue was mounted on the stage of a Zeiss inverted confocal laser scanning microscope (model 510 or 410) and viewed through a x40 water immersion objective (numeric aperture = 1.2). A 488-nm laser line (Ar ion laser), a 505-nm dichroic filter, and a 515-nm long-pass emission filter were used. Low laser intensity was used to avoid photobleaching. With the photomultiplier gain set to give a subepithelial space fluorescence intensity of 150–200 (full scale, 255) in control tissue loaded with 2 μM FL-MTX, autofluorescence of unloaded tissue was undetectable. From each piece of tissue under investigation, 10–15 areas of epithelium and adjacent vasculature were selected for measurement. Image acquisition and analysis have been described previously in detail (2).

[3H]MTX uptake. Each lateral choroid plexus (~1 mg) was incubated with shaking at 37°C in continuously gassed (95% O2 -5% CO2) aCSF containing 1 μM [3H]MTX and the indicated additions. After incubation, tissue was removed, rinsed briefly, weighed, and processed for liquid scintillation counting. Tissue accumulation of MTX was calculated from disintegrations per minute per milligram tissue wet weight and medium specific activity.

Statistics. Figures 1–10 show data from single experiments using tissue from six to eight animals; these are representative of two to four replicate experiments. Data are presented as means ± SE. Means were compared using Student’s t-test. Values were deemed to be significantly different at P < 0.05.

RESULTS

FL-MTX transport. Figure 2 shows transmitted light and confocal micrographs of choroid plexus tissue after 45 min incubation in aCSF containing 1 μM FL or 2 μM FL-MTX. In the transmitted light images, the apical brush-border membrane appears as a refractile element between cells and medium (Fig. 2, A and C). At high magnification, the epithelial cells are seen to contain a large central nucleus and smaller vesicular organelles (2). Beneath the epithelium is a distinct subepithelial space and blood vessels containing erythrocytes. Unlike most other capillaries in the brain, those in choroid plexus are fenestrated and thus the endothelium is no barrier to exchange of solutes between the vasculature and the interstitium. As shown previously (2), FL, accumulated in both the epithelial cells and the subepithelial/vascular spaces, with fluorescence intensities in vascular/subepithelial spaces > cells > medium (Fig. 2B). The pattern of FL-MTX fluorescence was clearly different, with levels in the cells slightly lower than the medium and levels in vascular/subepithelial spaces substantially higher than the medium (Fig. 2D). The only cellular element exhibiting FL-MTX fluorescence higher than the medium was the apical plasma membrane. In Fig. 2, B and D, erythrocytes can be seen within the blood vessels as areas of very low fluorescence, indicating exclusion of FL and FL-MTX from these cells or quenching of fluorescence by Hb. In spite of the low fluorescence within erythrocytes, spaces between and around these cells are highly fluorescent, with the fluorescence intensity being roughly equivalent to that seen in the subepithelial space.

Figure 3 shows the time course of FL-MTX accumulation in cells and vascular/subepithelial spaces. FL-MTX accumulated rapidly in the vascular/subepithelial compartment, with steady-state levels attained within 30 min. Although initial accumulation in the vascular/subepithelial compartment was rapid, a line drawn through the first two points intersects the time axis at 3 min, suggesting an initial delay in filling this compartment that may reflect the time required for the compound to cross the epithelium. The time course of cellular accumulation roughly paralleled vascular/subepithelial accumulation, but at a considerably lower level (Fig. 3). For comparison, medium fluorescence in this experiment averaged 25 units. At steady state, fluorescence in the vascular/subepithelial compartment was fivefold higher than the medium, and cellular fluorescence was only ~40% of medium fluorescence. Thus average fluorescence in the vascular/subepithelial compartment was at least an order of magnitude higher than in the cells, indicating potent basolateral efflux.

FL-MTX transport from bath to vascular/subepithelial spaces was dependent on metabolism and sensitive to replacement of medium Na. Inhibiting metabolism with NaCN reduced steady-state FL-MTX accumulation in the vascular/subepithelial spaces by ~80% (Fig. 4); cellular accumulation was not affected (data not shown). Decreasing medium Na...
replacement with N-methylglucamine or choline) caused a progressive decrease in FL-MTX accumulation in the vascular/subepithelial spaces (Fig. 4). In Na-free medium, transepithelial transport was reduced by 80%. Consistent with this, 1 mM ouabain substantially reduced FL-MTX accumulation. However, increasing medium K from 4.7 to 47 mM had no effect (Fig. 4). It was previously demonstrated that increasing medium K by an order of magnitude both depolarized choroid plexus cells by \( \sim 40 \) mV, decreased organic cation uptake (23), and blocked FL exit from the cells at the basolateral membrane (2). Together, the present data indicate that transepithelial FL-MTX transport across the epithelium was metabolism driven and Na dependent, but not PD sensitive.

Steady-state FL-MTX accumulation in both the cellular and vascular/subepithelial compartments saturated with increasing medium substrate concentration (Fig. 5). For both compartments, FL-MTX accumulation was a function of substrate concentration at low concentrations, but clearly reached a plateau at concentrations \( >5 \) \( \mu \)M. Note that parallel experiments at lower photomultiplier gain settings confirmed saturation of vascular/subepithelial space FL-MTX accumulation at 5–10 \( \mu \)M substrate concentrations (data not shown).

To begin to identify transporters responsible for transepithelial transport, we measured the effects of several compounds on steady-state FL-MTX accumulation in choroid plexus. Compounds were chosen based on their abilities to interact with Oats currently known to be expressed in the tissue, i.e., Oat1, Oat3, Oatp3, Mrp1, and Oatp2 (11, 12, 16, 19, 20). Note that because of the wide specificity limits of these Oats, it is difficult to identify compounds that interact specifically with only one transporter. Possible exceptions to this generalization are digoxin for Oatp2 and MK-571 (low affinity) for Mrp1 (16, 18). Figure 6 shows the effects of several organic anions on steady-state accumulation of FL-MTX in the vascular/subepithelial spaces. At 10 and 50 \( \mu \)M, MTX significantly reduced transepithelial transport. Probenecid, which affects transport on Oats, Oatps, and Mrps (16), inhibited at 100 \( \mu \)M. p-Aminohippurate (PAH), which interacts with Oat1 and Oat3, but not with Oatps and only weakly with Mrps (16, 19), was a poor inhibitor of transport. It was ineffective at 100 \( \mu \)M and only inhibited by \( \sim 30\% \) at 1 mM.

Dose-response data for four additional inhibitors are shown in Fig. 7. Two patterns of inhibition are evident. Estrone sulfate, a compound transported by Oat3, Oatps, and Mrps (9, 11, 16, 19), and leukotriene C\( _4 \) (LTC\( _4 \)), a high-affinity substrate for Mrps (16) and Oatps (10), decreased vascular/subepithelial accumulation of FL-MTX in a concentration-dependent fashion but did not affect cellular accumulation (Fig. 7, A and B). Digoxin, a high-affinity substrate for Oatp2 (16), and MK-571, a selective inhibitor of Mrps (16), also decreased vascular/subepithelial accumulation of FL-MTX in a concentration-dependent fashion (Fig. 7, C and D). However, both compounds significantly increased cellular accumulation. For digoxin, this increase was seen with concentrations as low as 0.01 \( \mu \)M, which is consistent with the known transport properties of Oatp2.
0.1 μM. With MK-571, significant increases were found with 50 and 100 μM. With 300 μM MK-571, both vascular/subepithelial accumulation and cellular accumulation were reduced substantially. Apparently, digoxin and MK-571 (unlike the other organic anions tested in Figs. 6 and 7, A and B) were more potent inhibitors of basolateral FL-MTX efflux than of apical uptake, and, when efflux was partially blocked, concentrative cellular accumulation became evident.

Using 10 μM digoxin and 100 μM MK-571 as tools to partially block efflux and thus enhance cellular accumulation, we could then begin to characterize the mechanism of apical uptake. In the presence of 10 μM digoxin, cellular accumulation of FL-MTX was nearly abolished by 200 μM probenecid. Replacement of medium Na had a similar inhibitory effect (Fig. 8A). In the presence of 100 μM MK-571, cellular accumulation of FL-MTX was reduced by ~90% by 200 μM probenecid, 50 μM estrone sulfate, and 1 μM LTC₄ (Fig. 8B). Consistent with a two-step transport mechanism, all of these treatments further reduced accumulation of FL-MTX in the vascular/subepithelial compartment. Thus FL-MTX accumulation at the apical membrane was indeed saturable, concentrative, specific, and Na dependent. In the absence of digoxin or MK-571, these characteristics of apical transport appeared to be masked by a particularly avid efflux mechanism at the basolateral membrane. Even at steady state, basolateral efflux maintained a

Fig. 3. Time course of FL-MTX accumulation in epithelial (cells) and vascular/subepithelial (vessels) compartments of choroid plexus. Tissue was incubated in medium with 2 μM FL-MTX, and at each indicated time confocal images were acquired. Mean fluorescence intensity in each compartment was measured as described in MATERIALS AND METHODS. The broken line indicates the fluorescence intensity of the medium. Each point represents the mean value for 8–10 measurements; variability is shown as SE bars.

Fig. 4. Effects of metabolic inhibition and ionic disruption on steady-state FL-MTX accumulation by choroid plexus. Tissue was incubated in medium with 2 μM FL-MTX without (control) and with the indicated modifications. After 45 min, confocal images were acquired. Shown are mean values for 12–15 measurements of fluorescence intensity in vascular/subepithelial (vessels) compartments; variability is given by SE bars. *Significantly lower than controls, P < 0.01.

Fig. 5. Effect of increasing medium FL-MTX concentration on steady-state accumulation in choroid plexus. Tissue was incubated in medium with 1–10 μM FL-MTX, and, after 45 min, confocal images were acquired. Shown are mean values for 10–15 measurements of fluorescence intensity in cellular and vascular/subepithelial (vessels) compartments; variability is given by SE bars. Inset: cellular fluorescence values plotted on an expanded scale.

Fig. 6. Inhibition of steady-state vascular/subepithelial accumulation of FL-MTX by organic anions. Tissue was incubated in medium with 2 μM FL-MTX without (control) and with the indicated additions. After 45 min, confocal images were acquired. Shown are mean values for 13–20 measurements of fluorescence intensity in the vascular/subepithelial compartment; variability is given by SE bars. *Significantly lower than controls, P < 0.05; **significantly lower than controls, P < 0.01. None of these compounds affected cellular fluorescence levels (data not shown).
very low cellular level of FL-MTX while concentrating the compound in the vascular/subepithelial space.

Although organic anions in combination with MK-571 reduced FL-MTX accumulation in both tissue compartments, a different result was found when digoxin was used in combination with MK-571. Figure 8B shows that incubating tissue in medium containing MK-571 plus digoxin reduced vascular/subepithelial accumulation of FL-MTX and increased cellular accumulation compared with tissue exposed to MK-571 alone. Because MK-571 and digoxin likely interact with different basolateral transporters, this result suggests that FL-MTX efflux is mediated by both Oatp2 and Mrp1.

MTX transport. It is of interest to compare the present findings using confocal imaging with those obtained using radiotracer methodology. Figure 9 shows the time course of uptake of 1 μM [3H]MTX by rat choroid plexus. Initially, uptake was rapid and linear with time; at times >5 min, uptake was nonlinear, suggesting an approach to steady state. Even at times as short as 5 min, accumulation of MTX by the tissue was concentrative, with calculated tissue-to-medium ratios at steady state approaching 20 (for 1 μM MTX, 1 pmol/mg tissue is equivalent to an uncorrected tissue-to-medium ratio of unity). Incubating tissue in low-Na medium (26 mM Na rather than 128 mM Na) caused a significant reduction in MTX uptake at 10, 30, and 60 min (Fig. 9). Uptake of MTX was also sensitive to inhibition by other organic anions. PAH, FL-MTX, and probenecid reduced uptake significantly and, as with FLMTX (above), estrone sulfate was a particularly good inhibitor (Fig. 10). Interestingly, 1 μM LTC4 did not inhibit, 1.0 μM digoxin was without effect, but 10 μM digoxin significantly reduced MTX uptake. Based on inhibition by 50 μM estrone sulfate, at least 85% of total MTX uptake at 15 min was mediated. Raising medium K from 4.7 to 47 mM did not significantly alter MTX uptake. Thus, in agreement with present experiments using FL-MTX, MTX uptake was concentrative, specific, Na-dependent, and mediated but not potential dependent. However, transport did not appear to be affected by LTC4 or by 1 μM digoxin.

DISCUSSION

A crucial obstacle to understanding xenobiotic transport in epithelia, such as renal tubule and choroid plexus, is correlating findings on transporter expression obtained from molecular/immunological studies with data on transport of individual compounds in the intact tissue or in vivo. This is particularly difficult for solutes that are handled by multiple transporters in tissues that are morphologically complex. For these solutes and tissues, tools are needed to define and characterize the separate components of transport and identify the molecular basis for each component. We recently utilized the power of quantitative, confocal microscopy-based techniques to probe in intact, living choroid plexus organic anion transport mechanisms at the cellular and subcellular levels (2). We found that transport of FL across the epithelium involved the following two concentrative steps: 1) Na-dependent uptake at the apical (CNS side) plasma membrane and 2) mediated, PD-driven efflux from the cells at the basolateral membrane. Based on findings with an Oat3 knockout mice (20, 21) and a recent reassessment of Oat3 energetics in Xenopus oocytes and rat kidney (19) and choroid plexus (Miller DS and Lowes S, unpublished observations), the Na-dependent, apical transporter for FL in choroid plexus appears to be Oat3. The PD-driven basolateral transporter remains to be identified.

In the present study, we used a similar approach to characterize the transport of a large, fluorescent organic anion, FLMTX (900 Da), across rat choroid plexus and found a more complicated picture. As with FL, transepithelial transport of FL-MTX was specific (reduced by other organic anions), concentrative, metabolism driven (inhibited by NaCN), and Na dependent. Unlike basolateral efflux of FL, which is blocked by high K, raising medium K by an order of magnitude had no
effect on FL-MTX transport. Uptake of [3H]MTX measured by scintillation counting was also specific, concentrative, and Na dependent, but it was not clear where in the tissue nonfluorescent MTX accumulated. MTX did inhibit FL-MTX transport, and FL-MTX did inhibit MTX transport, indicating that they shared at least one transporter. However, there were some disparities in inhibitor profiles (compare, e.g., the effects of digoxin and LTC4 in Figs. 7 and 10), suggesting differences in the ways that the tissue handled MTX and FL-MTX.

The steady-state tissue distribution of FL-MTX was clearly different from that found for FL, implying different mechanisms of transport. For FL, confocal micrographs showed the steady-state distribution to be vascular/subepithelial spaces > cells > medium (2). In contrast, the distribution of FL-MTX was vascular/subepithelial spaces > medium > cells (present study). Thus, based on tissue distribution of the substrates, it was clear that two concentrative steps in series drove transepithelial FL transport, but it was not clear whether FL-MTX transport involved mediated steps at both membranes or just at the basolateral membrane.

Experiments that tested the metabolism and ion dependence of FL-MTX transport provided no evidence for a two-step mechanism. That is, although exposure to NaCN and Na replacement greatly reduced vascular/subepithelial accumulation, these treatments did not alter cellular accumulation. This was also true for most of the organic anions that inhibited transepithelial transport. The first evidence that apical FL-MTX uptake might be mediated came from the observation that steady-state cellular accumulation appeared to saturate with medium substrate concentration. This could only have happened if apical uptake were mediated. However, it was when we tested digoxin and MK-571 as inhibitors that convincing evidence was found for a two-step transport mechanism. Both drugs increased cellular FL-MTX accumulation and decreased vascular/subepithelial accumulation. With 10 μM digoxin and 100 μM MK-571, mean cellular and vascular/

Fig. 8. Effects Na replacement and organic anions on steady-state FL-MTX accumulation by digoxin-treated (A) or MK-571-treated (B) choroid plexus. Tissue was incubated in medium with 2 μM FL-MTX without (control) and with the indicated additions. After 45 min, confocal images were acquired for later analysis. Shown are mean values for 12–17 measurements of fluorescence intensity in cellular and vascular/subepithelial (vessels) compartments; variability is given by SE bars. **Vessels significantly different from 10 μM digoxin or 100 μM MK-571 alone, P < 0.01. †Cells significantly different from digoxin or MK-571 alone, P < 0.05. ††Cells significantly different from digoxin or MK-571 alone, P < 0.01. Dig, digoxin; Prob, probenecid; ES, estrone sulfate.

Fig. 9. Time course of MTX accumulation in choroid plexus. Tissue was incubated in control and low-Na medium with 1 μM [3H]MTX. Shown are mean values for 4–6 intact plexuses; variability is given by SE bars. **Significantly different from controls, P < 0.01.

Fig. 10. Inhibition of steady-state MTX accumulation by organic anions and digoxin. Tissue was incubated for 15 min in medium with 1 μM [3H]MTX without (control) and with the indicated additions. Shown are mean values for 4–6 intact choroid plexuses; variability is given by SE bars. *Significantly different from control, P < 0.05. **Significantly different from controls, P < 0.01.
subepithelial FL- MTX accumulation were roughly equivalent to 50% of control vascular/subepithelial accumulation but exceeded medium FL- MTX by a factor of three to four. These results are consistent with digoxin and MK- 571 blocking FL- MTX efflux from the cells. Because increased cellular accumulation was accompanied by decreased vascular/subepithelial accumulation, it is likely that these drugs blocked basolateral efflux.

With vascular/subepithelial accumulation reduced by digoxin or MK- 571, concentrative uptake at the apical membrane was revealed and could be characterized. When tissue was incubated in medium containing digoxin plus probenecid, cellular and vascular/subepithelial accumulation of FL- MTX were substantially reduced compared with tissue exposed to digoxin alone, indicating mediated uptake at the apical membrane. Moreover, in the presence of digoxin, removal of medium Na greatly reduced cellular and vascular/subepithelial accumulation of FL- MTX, showing that apical uptake was Na dependent. Similarly, when tissue was incubated in medium containing MK- 571 plus probenecid, estrone sulfate, or LTC4, cellular and vascular/subepithelial accumulation of FL- MTX were substantially reduced compared with tissue exposed to MK- 571 alone. Thus, in the absence of digoxin or MK- 571, concentrative, mediated, and Na-dependent uptake of FL- MTX at the apical membrane was hidden by avid pumping of the compound from the cells in the vascular/subepithelial space.

Which transporters are responsible for transepithelial FL- MTX transport in choroid plexus? Although several Oats are expressed in the tissue, these possess greatly overlapping substrate specificities, and potent inhibitors of one, e.g., estrone sulfate and LTC4, cellular and vascular/subepithelial accumulation of FL- MTX were substantially reduced compared with tissue exposed to MK- 571 alone. Thus, in the absence of digoxin or MK- 571, concentrative, mediated, and Na-dependent uptake of FL- MTX at the apical membrane was hidden by avid pumping of the compound from the cells in the vascular/subepithelial space.

The present data provide some guidance in further narrowing down the list of possibilities. Note that digoxin and MK- 571 proved to be particularly useful tools in the present study because they were more potent inhibitors of basolateral efflux than of apical uptake. Other compounds tested have not shown this property, but other organic anions clearly inhibited FL- MTX transport. This means that each of the other inhibitors had a greater effect on uptake than on efflux. Assuming that inhibitor potency reflects specificity of the apical FL- MTX process, we are clearly looking for transporters that are driven directly or indirectly by Na, PD insensitive, and sensitive to inhibition by MTX, estrone sulfate, PAH, and LTC4. Because most Oats are remarkable in their ability to handle a wide range of anionic compounds, identification of the apical transporter(s) for FL- MTX will not be possible using specificity data alone. Available evidence points to Oatp2 and Mrp1 on the basolateral membrane as being the transporters responsible for basolateral FL- MTX efflux. Two findings indicate that FL- MTX efflux from the cells at the basolateral membrane is mediated. First, depolarizing the cells with high K had no effect on transport. If efflux of the organic anion was a result of simple diffusion (or even facilitated diffusion), it should have been reduced by depolarization. This was not the case. Second, digoxin and MK- 571 reduced vascular/subepithelial accumulation, while at the same time increasing cellular accumulation of FL- MTX, consistent with inhibition of efflux at the basolateral membrane mediated by Oatp2 and Mrp1. Digoxin is a potent inhibitor of transport on Oatp2 (12), which can operate as an organic anion exchanger and as a uniporter (10). For Oatp2 to mediate FL- MTX efflux in choroid plexus, transport must be electrically silent, e.g., organic anion exchange. Such exchange would require a counter ion with a concentration gradient that is appropriately directed to drive FL- MTX efflux. It is not clear at present what the physiological counter ion would be.

MK- 571 is a selective inhibitor of Mrps, which are ATP-driven efflux pumps and thus not responsive to changes in PD. Although MTX is not apparently a substrate for transport on Oatp2 (12), it is transported by a number of Mrp isoforms (16), and FL- MTX is clearly a potent inhibitor and substrate for Mrp2 (22). Because Mrp1 and Mrp2 do not differ radically in specificity, it is likely that, in rat choroid plexus, Mrp1 (along with Oatp2) mediates FL- MTX efflux at the basolateral membrane.

In summary, confocal imaging demonstrated that FL- MTX accumulated to high levels in the vascular/subepithelial space of rat choroid plexus, but not in the epithelial cells. Nevertheless, transepithelial transport involved two mediated steps: Na-dependent uptake at the apical membrane and potent, electroneutral efflux at the basolateral membrane. The transporters responsible for uptake remain to be identified, but efflux is most likely mediated by Oatp2 and Mrp1.

REFERENCES


