Immunohistochemical and Functional Characterization of PEPT, OCT, ATB\(^{0,+}\), and MCT Drug Transporters in Human Ocular Tissues

Rajendra S. Kadam, Sunil K. Vooturi, and Uday B. Kompella

Department of Pharmaceutical Sciences (R.S.K., S.K.V., and U.B.K.)

Departments of Ophthalmology and Bioengineering (U.B.K.)

University of Colorado Anschutz Medical Campus, Aurora, CO 80045
Running Title: Drug Transporters in Human Ocular Tissues

Corresponding author: Uday B. Kompella, Ph.D., Department of Pharmaceutical Sciences, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado Anschutz Medical Campus, 12850 E. Montview Blvd., Aurora, CO 80045 (Phone: (303) 724-4028; Fax: (303) 724-4666; E-mail: uday.kompella@ucdenver.edu)

Number of text pages: 38
Number of tables: 4
Number of figures: 11
Number of references: 42
Number of words in abstract: 248
Number of words in introduction: 732
Number of words in discussion: 1499

List of abbreviations:
PEPT: peptide transporter; OCT: Organic cation transporters; ATB\textsuperscript{0,+}: Neutral and basic amino acid transporter; MCT: Monocarboxylate transporters; SCR: Sclera-choroid-retinal pigment epithelium-retina; RPE: Retinal pigmented epithelium; SCRPE: Sclera-choroid-retinal pigmented epithelium; SLC: Solute carrier transporters; H&E: hematoxylin and eosin;
ABSTRACT

Since there is paucity of information on solute transporters in human ocular tissues, the aim of this study was immunohistochemical and functional characterization of PEPT, OCT, ATB\textsuperscript{0,+}, and MCT transporters in human ocular barriers. Immunohistochemical localization of transporters was achieved using 5 µm thick paraffin embedded sections of the whole human eyes. In vitro transport studies were carried out across human cornea and sclera-choroid-RPE (SCRPE) using a cassette of specific substrates in the presence and absence of inhibitors in order to determine the role of transporters in transtissue solute delivery. Immunohistochemistry showed the expression of PEPT-1, PEPT-2, ATB\textsuperscript{0,+}, OCT-1, OCT-2, MCT-1, and MCT-3 in human ocular tissues. PEPT-1, PEPT-2, OCT-1, MCT-1, and ATB\textsuperscript{0,+} expression was evident in cornea, conjunctiva, ciliary epithelium, and neural retina. Further, expression of PEPT-1, PEPT-2, and OCT-1, was evident in choroid tissue as well. OCT-2 expression could be visualized in the corneal and conjunctival epithelia, while MCT-3 expression was confined to RPE layer. While OCT-2 expression was evident in conjunctival blood vessel walls, PEPT-1, PEPT-2, and OCT-1 were expressed in the choroid. Preliminary transport studies indicated inward transport of Gly-Sar (PEPT substrate), MPP\textsuperscript{+} (OCT substrate), and L-tryptophan (ATB\textsuperscript{0,+} substrate) across cornea as well as SCRPE. For phenylacetic acid (MCT substrate), transporter mediated inward transport across cornea and outward transport across SCRPE was evident. Thus, PEPT, OCT and ATB\textsuperscript{0,+} are influx transporters present in human ocular barriers and they can potentially be used for transporter guided retinal drug delivery after topical, transscleral, and systemic administrations.
INTRODUCTION

Intraocular drug delivery is a major challenge due to unique, protective barriers present. Topical route, the most common method of ocular drug delivery, typically results in less than 5% bioavailability in the anterior segment eye tissue and less than 0.05% in the posterior segment eye tissues including the retina, due to the presence of ocular surface barriers including corneal and conjunctival epithelia (Kompella et al., 2010). Systemic route of drug delivery to the retina is limited by outer and inner blood-retinal-barriers consisting of retinal pigment epithelium (RPE) and retinal endothelial layer, respectively (Sunkara G and Kompella UB, 2003). Topically or systemically administered drug must be absorbed through ocular barriers to reach the target tissues. Low molecular weight drugs get absorbed through biological barriers primarily via passive diffusion and carrier mediated transport (Sugano et al., 2010). One approach to enhance drug delivery to the target site is the use of body’s own biological mechanisms such as plasma membrane transporters. Mammalian cells express various transporters in the plasma membrane to allow cellular delivery of essential nutrients from the extracellular space (Ganapathy and Ganapathy, 2005). These transporters can potentially be utilized for the delivery of drug molecules that are structurally similar to the transporter substrates.

There are various examples in the literature, showing that the transport of small drug molecules across plasma membrane can occur by solute carrier transporters (Kimura et al., 2005; Giacomini et al., 2010). Further, various attempts were made in the literature to enhance the delivery of poorly permeable drugs by making them substrates for a particular transporter through prodrug derivatization (Gynther et al., 2008; Peura et al.,
One key example of transporter guided prodrug delivery is valacyclovir, a L-valine ester of acyclovir, which has 3- to 5-fold greater oral bioavailability as compared to the parent acyclovir (Weller et al., 1993). Transport of valacyclovir across human intestinal epithelium is mediated through oligopeptide (Guo et al., 1999) and amino acid transporters (Hatanaka et al., 2004).

Transporter guided drug delivery has been extensively characterized for oral and brain delivery. On the other hand, there is a dearth of knowledge in the literature for characterization and utilization of drug transporters in ocular drug delivery. Pioneering research by Drs. Vincent. Lee, Vadivel Ganapathy, Ashim Mitra, and Kenichi Hosoya, showed the presence of solute transporters in ocular barriers using isolated cell cultures and preclinical animal models. However, till date there is no report showing the functional characterization of drug transporters in human ocular tissues. Zhang et al. showed the mRNA expression of various influx and efflux drug transporters in cornea, iris-ciliary body, and choroid-retina (Zhang et al., 2008). It is well known that there is lack of correlation between mRNA and protein levels (Gygi SP et al., 1999), raising uncertainty about the transporter proteins that are active in human eye tissues.

This study characterized four solute carrier (SLC) transporters including peptide transporters (PEPT), amino acid transporter (ATB0,+), organic cation transporters (OCT), and monocarboxylate transporters (MCT) in human ocular tissues. Previous reports showed that the PEPT, OCT, MCT and ATB0,+ are influx drug transporters in rabbit ocular tissues and can be used for ocular drug delivery (Horibe et al., 1998; Ueda et al., 2000; Anand and Mitra, 2002; Hatanaka et al., 2004). Of these four transporters, characterization of transporter expression at the protein level was only reported for MCT
in human RPE and retina (Philp et al., 2003). Zhang et al. (Zhang et al., 2008) and Ocheltree et al. (Ocheltree et al., 2003) have characterized the expression of PEPT in human choroid-retina and cornea at mRNA level. For ATB$^0,+$, while data is available with preclinical animal models (Anand and Mitra, 2002; Hatanaka et al., 2004; Jain-Vakkalagadda et al., 2004), there is no report available showing ATB$^0,+$ expression at the protein levels in human ocular tissues. Further, to our knowledge, there are no reports available in the literature on functional characterization of any of these transporters in human ocular barriers.

Thus, in order to address the paucity of human ocular tissue data for drug transporters, this study characterized the expression of PEPT, ATB$^0,+$, OCT, and MCT transporter proteins in human ocular tissues including cornea, conjunctiva, ciliary epithelium, retinal pigment epithelium, choroid, and neural retina using immunohistochemistry. Further, the activity and polarity of transporters were determined by measuring the transport of specific substrates across isolated human sclera-choroid-RPE and cornea in the presence and absence of transporter specific inhibitors.
MATERIALS AND METHODS

Materials

MPP⁺ iodide (≥ 98.0%), α-methyl-DL-tryptophan (98%), phenylacetic acid (98%), L-tryptophan (> 98%), Gly-Sar, metformin (~97%), nicotinic acid sodium salt, nadolol (~98%) and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). H-Pro-Phe-OH (> 99%) was purchased from Bachem (Torrance, CA). HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ). Ammonium formate (99.9%) was purchased from Fluka BioChemika (USA). All primary antibodies except ATB⁰⁺ antibody were purchased from Santa Cruz Biotech, (Santa Cruz, CA). ATB⁰⁺ antibody was purchased from Medical and Biological Laboratories, Japan. All other chemicals and reagents used in this study were of analytical reagent grade.

Human Eyes and Tissue Specimens

For transport studies, human cadaver eyes were obtained from the Rocky Mountain Lions Eye Bank (Aurora, CO) within 48 hr of death. For immunohistochemical analysis of transporters, human ocular tissue specimens were obtained from the archives of University of Colorado, Anschutz Medical Campus eye pathology laboratory. Procedures were in compliance with the Declaration of Helsinki Statement for the research involving the use of human tissues and the protocol was approved by Institutional Review Board of University of Colorado. Summary of patient data including age, sex, condition of eye and reason for death are provided in Table 1. For the transport study, eyes were immediately used upon arrival. For immunohistochemistry, formalin-
fixed paraffin embedded 5 µm thick sections of whole human eyes were obtained from
the archives of the University of Colorado Eye Pathology Laboratory.

**Immunohistochemical Analysis**

For immunohistochemical staining, formalin-fixed paraffin embedded 5 µm thick
sections were obtained from the whole human eye and mounted on (3-aminopropyl)
triethoxysilane-treated slides. Slides were deparaffinized in xylene for 20 min to remove
the embedding paraffin media and washed with absolute ethanol. Slides were gradually
rehydrated using series of 5 min washes, including 95, 90, 70, and 50% alcohol and
distilled water. Endogenous peroxide activity was blocked by incubating slides with 3%
H₂O₂ in absolute methanol for 15 min at 37 °C. Whenever necessary antigen retrieval was
performed by incubating the slides in boiling 10 mM citrate buffer (pH 6.0) or 10 mM
Tris-HCL containing 1 mM EDTA (pH 9.0) at 95 °C for 20 min. After antigen retrieval,
slides were washed and permeabilized with phosphate buffer saline (PBS) containing 0.1
% Triton X-100 (PBS-T). Nonspecific antibody binding was blocked by incubating the
slides with blocking buffer (1.0 % BSA and 10 % goat serum in PBS). Tissue sections
were then incubated with appropriate dilution of primary antibody in PBS-T at 37 °C for
1 hr or at 4 °C overnight. Summary of primary antibody dilution, incubation condition,
antigen retrieval procedure, secondary antibody, and detection system used are provided
in Table 2. After incubation with primary antibody, sections were washed three times
with PBS-T and then incubated with appropriate dilution of alkaline phosphates linked-
secondary antibody (Leica; Bond™ Polymer Refine Red Detection) in Tris buffer saline
for 30 min. Sections were washed with PBS-T and then stained and visualized using the
VECTOR® Red alkaline phosphatase detection system (Vector Laboratories; Vector®
Red Alkaline Phosphatase Substrate Kit I). Slides were counterstained with hematoxylin (Auto Hematoxylin; Open Biosystems) for 30 seconds to stain the nuclei. For controls, the sections were processed same as above after omitting the incubation step with primary antibody.

**In Vitro Transport across Human Cornea and Sclera-choroid-RPE**

In vitro transport studies across human cornea and sclera-choroid-RPE (SCRPE) were carried out according to a previously published method (Kadam et al., 2011) using the cassette dosing approach. A cassette of drug transporter substrates including Gly-Sar (PEPT), L-tryptophan (ATB0,+)，MPP+ (OCT), and phenylacetic acid (MCT) at concentration of 100 µM in assay buffer was prepared. Briefly, the human eyes were washed with assay buffer and cleaned from muscles and conjunctiva tissues, and the anterior and posterior parts were separated by giving a circumferential cut behind the limbus. A small sclera part was left with cornea, which helps in mounting of cornea on Ussing chambers. Neural retina was separated from the choroid-RPE by filling the eye cup with assay buffer, and floating retina was collected. After separation of retina, the eye cup was divided into two rectangular pieces (~1.5 X 1.5 cm) of sclera-choroid-RPE. Isolated tissues were mounted in modified Ussing chambers (Navicyte, Sparks, NV) such that the episcleral side of SCRPE or epithelial side of cornea was facing the donor side and retinal side or endothelial side of cornea was facing the receiver side. To study the effect of directionality on transport, one set of Ussing chambers were mounted such that the sclera side was facing the donor side and another set was mouthed such that choroid-RPE was facing the donor side. Due to limited availability of human eyes, the effect of directionality on transport across cornea was not evaluated. Chambers were filled with
1.5 ml of assay buffer with (donor side) or without (receiver side) the cocktail of drug transporter substrates. To study the effect of transporter inhibitors, a cocktail mixture (500 μM each) of transporter inhibitors was added to both donor and receiver sides. Summary of specific transporter substrates and inhibitors used for the transport study are provided in Table 3. During the transport study, the bathing fluids were maintained at 37 °C using a circulating warm water bath and the assay buffer was maintained at pH 7.4 using 95% air - 5% CO₂ aeration. Samples were collected (200 µL) from the receiver side every hr for 6 hr and the lost volume was compensated with fresh assay buffer pre-equilibrated at 37 °C. Drug levels were analyzed using LC-MS/MS. Permeation data was corrected for dilution of the receiver solution due to sample volume replenishment.

**LC-MS/MS Analysis**

Analyte concentrations in the transport study samples were analyzed using LC-MS/MS method after 5-fold dilution with acetonitrile to reduce the salt concentrations. An API-3000 triple quadrupole mass spectrometry (Applied Biosystems, Foster City, CA, USA) coupled with a PerkinElmer series-200 liquid chromatography (Perkin Elmer, Walthm, Massachusetts, USA) system was used for analysis. A cassette analysis method was developed for simultaneous analysis of Gly-Sar, L-tryptophan, and MPP⁺. Phenylacetic acid was analyzed separately. Gly-Sar, L-tryptophan, and MPP⁺ were separated on a Supelco C-5 column (2.1 x 10 mm, 3 μm) using water containing 0.1 % formic acid (A) and acetonitrile: methanol (50:50 v/v) containing 0.1% formic acid (B) as mobile phase. A linear gradient elution at a flow rate of 0.3 ml/min with a total run time of 9 min was used. Phenylacetic acid was separated in normal phase separation mode using Obelisc-N silica column (2.1 x 10 mm, 3 μM) with 5 mM ammonium formate, pH
3.5 (A) and acetonitrile (B) as the mobile phase in linear gradient mode at a flow rate of 0.3 ml with a total run time of 6 min. Gly-Sar, L-tryptophan, and MPP* were analyzed in positive ionization mode with the following multiple reaction monitoring transitions: 147 → 90 (Gly-Sar); 205 → 188 (L-tryptophan); 170 → 128 (MPP*). Phenylacetic acid was analyzed in negative ionization mode with the following multiple reaction monitoring transitions: 135 → 91 (phenyl acetic acid).

Data Analysis

All values in this study were expressed as mean ± SD. Replicates in each experiment indicate the data from independent donors. Statistical comparisons between two groups were determined using independent sample Student’s *t*-test. Differences were considered statistically significant at *p* <0.05.
RESULTS:

Patient Demographics and H & E Staining of Human Eyes

Paraffin embedded 5 µm thick sections of whole human eyes were obtained from 4 patients. Demographic information of patients is summarized in Table 1. Eye sections were stained with hematoxylin and eosin (H&E) for anatomical assessment. Representative H&E images are shown in Figure 1. The H&E image of cornea showed intact multi-layered epithelium, followed by thick stroma with sparse fibrocytes (keratocytes), and single layer of endothelial cells. The H&E stain image of conjunctiva showed multilayer epithelium followed by stroma with larger number of fibroblasts and globet cells. Ciliary body showed the single layer of inner nonpigmented epithelial cells followed by outer pigmented epithelium, and ciliary muscles, which attach it to the sclera. The H&E section of sclera-choroid-retinal pigment epithelium-retina (SCR) showed all 8 distinguished layers of retina, single layer of retinal pigmented epithelium (RPE), choroid, and sclera.

Localization of PEPT-1 and PEPT-2 in Human Ocular Tissues

Four eyes from four donors were examined for PEPT transporter expression. A representative image of a negative control is shown in Figure 2. Of the two PEPT transporters, PEPT-1 staining was less abundant than PEPT-2 in all ocular tissues (Figure 3 and 4). As shown in Figure 3, PEPT-1 showed very light immunolabeling in the conjunctival and corneal epithelia. Nonpigmented ciliary epithelium showed more intense immunolabeling than the cornea and conjunctiva. With regards to the SCR, light immunolabeling of PEPT-1 was observed in the inner nuclear and ganglion cell layers and outer plexiform layer of the retina, RPE, and smooth muscles of choroidal blood
vessels (Figure 3). Interestingly, PEPT-2 showed very intense staining in all ocular tissues. In cornea and conjunctiva, PEPT-2 immunolabeling was confined only to the epithelial layers with uniform distribution throughout the epithelial layers. In the ciliary body, PEPT-2 immunolabeling was only observed in nonpigmented ciliary epithelium. In the SCR, PEPT-2 showed very strong labeling in the outer segment of the rod cells of the retina (Figure 4). PEPT-2 labeling was also observed in the ganglion cell layer of retina, RPE, and smooth muscles of choroidal blood vessels.

**Localization of ATB$^{0,+}$ in Human Ocular Tissues**

ATB$^{0,+}$ was expressed in human cornea, conjunctiva, ciliary body, retina, and RPE with staining confined near the nucleus for all tissues. As slides were counterstained with hematoxylin, and ATB$^{0,+}$ labeling was visualized using Poly-alkaline phosphate red; colocalization of red and blue signal showed a reddish brown color instead of red color. As shown in Figure 5, ATB$^{0,+}$ labeling was observed in the corneal and conjunctival epithelia as well as potentially fibrocytes in the stroma. Nonpigmented ciliary epithelium showed brighter ATB$^{0,+}$ staining than all other tissues. For SCR, ATB$^{0,+}$ labeling was observed in the inner and outer nuclear layer, ganglion cell layer, and RPE.

**Immunohistochemical Localization of OCT-1 and OCT-2 in Human Ocular Tissues**

In case of organic cation transporters, only OCT-1 showed immunostaining in neural retina. OCT-1 showed brighter staining in the corneal epithelium compared to conjunctival epithelium (Figure 6). In SCR, OCT-1 labeling was localized to the inner segment of photoreceptor cells and RPE layer. Light staining with OCT-1 was also present in the smooth muscles of choroidal blood vessels.
As shown in Figure 7, nonpigmented and pigmented ciliary epithelium and choroid-retina were devoid of OCT-2 labeling. In the cornea and conjunctiva epithelium, OCT-2 expression was localized more towards the outer layer of epithelium. In conjunctiva, OCT-2 labeling was also evident in the smooth muscles of conjunctival blood vessels.

Immunohistochemical Localization of MCT-1 and MCT-3 in Human Ocular Tissues

As shown in Figure 8, MCT-1 immunolabeling was evident in the basal cells of the epithelium of cornea and conjunctiva. Nonpigmented ciliary epithelium showed strong labeling with MCT-1 (Figure 8). For SCR, MCT-1 labeling was observed only in the inner limiting membrane, outer segment of photoreceptor cells, and RPE cell layer. In case of MCT-3, immunohistochemical labeling was only seen in the RPE layer (Figure 9).

Transport of Transporter Substrate Cassette across Human Sclera-choroid-RPE (SCRPE)

Transport of transporters specific substrates was carried out across human SCRPE for evaluation of functional activity of transporters in SCRPE barriers. Due to limited availability of human eyes, a cassette dosing approach was used to reduce the tissue usage and increase the throughput (Kadam and Kompella, 2009). The effect of directionality was evaluated to determine whether, a particular transporter is contributing to the influx of drug into the retina or efflux form the retina. As shown in Figure 10, transport of Gly-Sar (PEPT substrate), MPP⁺ (OCT substrate), and L-tryptophan (ATB³⁺ substrate) from sclera-to-retina direction was higher than the retina-to-sclera direction, which indicates that these transporters might act as influx transporters in retinal drug
delivery. For phenylacetic acid (MCT substrate), retina-to-sclera transport was higher than the sclera-to-retina transport, indicating that MCT might be an outward transporter, transporting molecules out from retina to the choroid (Figure 10D). Due to limited availability of human eyes, sample size was limited to n=2 for some of the directionality experiments. Samples were not collected at zero time point. However, for graphical representation of cumulative % transport data, transport at zero hour time point was assumed to be zero.

Further, sclera-to-retina transport was carried out in the presence and absence of inhibitors cocktail to evaluate the contribution of active transporter mediated transport in total transport across SCRPE. As shown in Figure 10, sclera-to-retina direction transport of Gly-Sar, MPP+, and L-tryptophan was significantly inhibited in the presence of inhibitors cocktail, which indicates that the transport of these molecules across human SCRPE is mediated through these drug transporters. In case of phenylacetic acid (PHA), sclera-to-retina direction transport of PHA was not inhibited in the presence of inhibitor, indicating that the transport of PHA is may not be mediated by the transporters investigated (Figure 10 D).

Transport of Transporter Substrate Cassette across Human Cornea

Due to the limited availability of human eyes, the effect of directionality on transport was not evaluated across the cornea. Transport of transporter substrate cassette across the cornea was evaluated in the presences and absence of inhibitors. As shown in Figure 11, the transport of MPP+, L-tryptophan, and phenylactetric acid across human cornea was inhibited in the presence of the inhibitors cocktail. The cumulative %
transport of Gly-Sar across human cornea was 10.2 %, and in the presence of Pro-Phe (PEPT inhibitor), it was reduced to 6.0 %.
DISCUSSION

Our results indicated the differential expression of PEPT, OCT, ATB\textsuperscript{0,+}, and MCT in human ocular tissues. All four transporters were found to be inward transporters in cornea and SCRPE, except for MCT, an outward transporter in SCRPE.

Previous gene expression studies of human ocular tissues showed strong expression of PEPT-2 and weak expression of PEPT-1 (Ocheltree et al., 2003; Zhang et al., 2008). Further, these studies showed the absence of PEPT-1 expression in human choroid-retina. In the current study, we observed weak expression of PEPT-1 in the choroidal smooth muscles, RPE, and inner nuclear layer of the retina. Although no report is available on expression analysis of PEPT transporters in human conjunctiva, Basu et al. (Basu et al., 1998) and Sun et al. (Sun, 1996) showed PEPT activity in the rabbit conjunctiva. We observed very strong expression of PEPT-2 and moderate expression of PEPT-1 in bulbar conjunctival epithelial cells. Results from in vitro transport studies confirmed the influx transport activity of PEPT in human SCRPE and cornea (Figures 10 and 11). Previous studies showed significant activity of PEPT transporters at pH 7.4 in rabbit cornea and conjunctiva (Basu et al., 1998; Anand and Mitra, 2002). As suggested by Smith et al. (Smith et al., 2004), local hydrogen gradient created by Na\textsuperscript{+}/H\textsuperscript{+} exchangers in the plasma membrane may also drive PEPT activity. Our results corroborate with published preclinical reports, which showed involvement of PEPT transporters in dipeptide transport across rabbit SCRPE and cornea (Anand and Mitra, 2002; Majumdar et al., 2005; Kansara et al., 2007).

ATB\textsuperscript{0,+} is the only transporter in mammalian cells which has the ability to concentrate cationic amino acids inside the cells using Na\textsuperscript{+}/Cl\textsuperscript{−} gradient and it can
transport 18 out of 20 amino acids (Ganapathy and Ganapathy, 2005). Immunohistochemistry showed ATB\(^{0,+}\) expression in several ocular tissues (Figure 5). Ubiquitous distribution of ATB\(^{0,+}\) in all nuclear layers of retina might be due to the high need of amino acids such as glycine for neurotransmission as well as protein synthesis. Glycine concentrations in neural retina is fivefold higher than the plasma (Okamoto et al., 2009), and glycine accumulates in the retina because of the highly concentrative capacity of ATB\(^{0,+}\) and glycine transporters (Ganapathy and Ganapathy, 2005; Okamoto et al., 2009). Results observed in our study are supported by earlier preclinical studies, which showed the involvement ATB\(^{0,+}\) in the transport of amino acid prodrugs of acyclovir and L-arginine across rabbit cornea (Jain-Vakkalagadda et al., 2004; Majumdar et al., 2009).

Although the expression of OCT isoforms tested in the current study was not as abundant as PEPT transporters, the cumulative % transport of Gly-Sar and MPP\(^+\) across SCRPE and cornea was comparable. With immunohistochemical analysis, we only tested the expression of OCT-1 and OCT-2. Previous reports showed abundant expression of OCTN-1, OCNT-2 and OCT-3 isoforms in human ocular tissues (Garrett et al., 2008; Zhang et al., 2008; Xu et al., 2010). MPP\(^+\) used in this study as a substrate for OCT transporter has broad specificity and interacts with both OCT as well as OCTN transporters (Jonker and Schinkel, 2004; Jong et al., 2011).

The last transporter we explored for characterization in human ocular tissues was monocarboxylate transporter (MCT). Although Philip et.al., (Philp et al., 2003) characterized the expression of MCT-1 and MCT-3 in human choroid-retina, no data was available on the expression of these transporters in other human ocular tissues. Gene expression analysis of rat ocular tissues showed that MCT-1 and MCT-3 are the most
abundant isoforms in ocular tissues (Chidlow et al., 2005). For MCT-1, we observed immunolabeling in photoreceptors cells, inner retinal layer, RPE, iris-ciliary body, and corneal and conjunctival epithelia (Figure 8). For MCT-3, immunostaining was confined to the RPE layer and no staining was observed in any other ocular tissues (Figure 9). Previous reports also showed immunolocalization of MCT-3 only in the RPE layer (Philp et al., 2003; Chidlow et al., 2005). We observed that MCT acts as an inward transporter in cornea and as an outward transporter in SCRPE. Retina is a metabolically highly active tissue and produces large amounts of lactic acid by aerobic metabolism of glucose (Hosoya et al., 2001; Chidlow et al., 2005). It is well know that the MCT-1 and MCT-3 in the retina and RPE act as outward transporters to remove lactate form subretinal space to the choroidal circulation and to maintain cellular homoeostasis (Hosoya et al., 2001; Chidlow et al., 2005). But in the cornea and conjunctiva, MCT acts as an influx transporter to reabsorb lactate form the tear fluid, where lactate is present at very high concentration (1 to 5 mM) (Horibe et al., 1998). A recent study also showed that the same isoform of MCT can act as an inward or outward transporter in hypothalamic glial cells depending upon the glucose and lactate concentrations available in the culture medium (Cortes-Campos et al., 2011). Facilitated bidirectional transport ability of the MCT transporter may be used to mediate the delivery of monocarboxylic acid drug molecules across the ocular barriers.

Transport of drug molecules across epithelial barrier is a two-step process, first uptake into epithelial cells followed by exit from the cell to the opposite side. We did not dissect these steps in the present study but assessed transtissue transport. However, we carefully selected the specific substrates and inhibitors based on the unique structural
requirements of individual transporters (Table 3). Dipeptide, Gly-Sar, is a well-known substrate for PEPT transporters. Biegel et al. reported the characterization of several hundred substrates/inhibitors of PEPT transporters using Gly-Sar as a control (Biegel et al., 2006). Gly-Sar is specifically transported by PEPT transporters and it is expected to not have any cross reactivity with ATB0,+, MCT, and OCT transporters. ATB0,+ cannot transport the dipeptide (Gly-Sar) owing to the requirement that α-COOH group of the amino acid should be either free acid or esterified, but it cannot be amidated (Umapathy et al., 2004). Tsuji et al. reported that MCT transporters do not bind to amino acids or dipeptides (Tsuji et al., 1994). Brandsch et al. reported the inhibitory effects of Pro-Phe against PEPT-1 and PEPT-2 transporters (Brandsch et al., 1999). Owing to the higher hydrophobicity of Pro-Phe, it has higher affinity than Gly-Sar for the transporter and hence, inhibits the transport of Gly-Sar effectively (Biegel et al., 2006). MPP+ is a widely used substrate of OCT transporters and it is transported by all OCTs including OCT-1, OCT-2, OCT-3, and OCTN (Jonker and Schinkel, 2004; Jong et al., 2011). Umehera et al. have reported that metformin inhibits the transport of MPP+ by OCT-1, OCT-2, and OCT-3 transporters (Umehera et al., 2007). Phenformin and cimetidine are more potent inhibitors of OCT than metformin; however, metformin was preferred since it does not interact with efflux transporters such as MDR and MRP (Pedersen et al., 2008). L-Tryptophan is not an exclusive substrate for ATB0,+, and it also transported by L and y+L type amino acid transporter system but known to have higher binding affinity to ATB0,+ than all other amino acids (Karunakaran et al., 2008). Karunakaran et al. have reported the inhibitory effect of α-methyl tryptophan, which is not transported by ATB0,+ (Karunakaran et al., 2008). L-lactic acid and other aliphatic monocarboxylates such as L-
propionic acid are most commonly used to characterize the MCT transporters. However, we used phenylacetic acid instead of aliphatic acids based on the fact that they have better detectability using LC-MS/MS. Phenylacetic acid and nicotinic acid are transported by MCT transporters and do not have any cross reactivity with ATB$^{0,+}$ and PEPT transporters (Tsuji et al., 1994).

Substrate and inhibitor concentrations were selected based on their reported Km/Ki/IC$_{50}$ values in literature. Reported Ki values of metformin for hOCT-1 and hOCT-2 are 493 and 289 µM, respectively (Umehara et al., 2007) and reported Ki values of Pro-Phe for PEPT-1 and PEPT-2 are 510 and 110 µM, respectively (Biegel et al., 2006).

Although the transporter substrates used in this study have a molecular weight ≤ 205, the transporters involved are likely to transport some larger molecules. Various large drug molecules such as cephalexin (MW 347.4), valsartan (MW 435.5), pindolol (MW 248.3), and oxaliplatin (MW 397.3) are transported by transporters (Giacomini et al., 2010). Along with dipeptides, PEPT-2 also transports cefadroxil, bestatin, and valacyclovir. OCT-2 transporter also transports drug molecules such as pindolol, ranitidine, amiloride, and oxaliplatin (Giacomini et al., 2010). Despite the carefully selection of transporter substrates and inhibitors, the interactions at the cellular level between inhibitors/substrates are complex and cross-reactivity cannot be ruled out.

In summary, this study for first time showed immunohistochemical and functional characterization of four drug transporters (PEPT, ATB$^{0,+}$, OCT, and MCT) in human ocular tissues. Out of the four transporters, PEPT, ATB$^{0,+}$, and OCT are influx drug transporters, showed ubiquitous distribution in ocular barriers, have wide substrate specificity, and have the potential to be utilized for transporter mediated intraocular drug...
delivery. MCT transporter acts as an inward transporter in cornea and outward transporter in SCRPE and can likely be used for the delivery of monocarboxylate drug molecules to the anterior segment after topical application.
ACKNOWLEDGEMENTS

We thank Mary Jo Garascia at the Ophthalmic Pathology Core Facility at University of Colorado, Anschutz Medical Campus for the preparation of histological sections and H & E staining of human eyes. We also thank Patsy Ruegg of IHCtech, LLC at Fitzsimmons BioScience Park for her help in immunohistochemistry of human ocular tissue samples.
Authorship Contributions

Participated in research design: Kadam, Vooturi, and Kompella.

Conducted experiments: Kadam.

Contributed new reagents or analytic tools: Kadam and Kompella.

Performed data analysis and/or interpretation: Kadam, Vooturi, and Kompella.

Wrote or contributed to the writing of the manuscript: Kadam, Vooturi, and Kompella.
REFERENCES


Hatanaka T, Haramura M, Fei YJ, Miyauchi S, Bridges CC, Ganapathy PS, Smith SB, Ganapathy V and Ganapathy ME (2004) Transport of amino acid-based prodrugs by the Na+- and Cl(-) -coupled amino acid transporter ATB0,+ and expression of


Footnotes

This work was supported by a research grant from National Institutes of Health National Eye Institute [Grants R01-EY018940, R01-EY017533].
FIGURE LEGENDS

**Figure 1.** Anatomical assessment of human ocular tissues. Human ocular tissue sections were stained with hematoxylin and eosin for morphological assessment. Numbers in sclera-choroid-retina refer to different layers: 1, inner limiting membrane; 2, ganglion cell layer; 3, inner plexiform layer; 4, inner nuclear layer; 5, outer plexiform layer; 6, outer nuclear layer; 7, inner segment of photoreceptor cell; 8 outer segment of photoreceptor cell; 9, retinal pigmented epithelium; 10, choroid; 11, sclera.

**Figure 2.** Representative negative control images for immunohistochemical localization of drug transporters in human ocular tissues. For control experiments, the sections were processed same as test samples except the incubation step with primary antibody was omitted. Nuclei were counterstained as blue with hematoxylin.

**Figure 3.** Representative figure of immunohistochemical localization of PEPT-1 in human ocular tissues. Arrowhead indicates the localization of PEPT-1 staining. Light staining of PEPT-1 was observed in epithelial layers of cornea and conjunctiva and nonpigmented epithelial layer of ciliary body. In sclera-choroid-retina, strong labeling of PEPT-1 was observed in outer plexiform layer and light staining was observed in RPE cell layer, smooth muscles of choroid blood vessels and ganglion cell layer. Nuclei were counterstained as blue with hematoxylin.

**Figure 4.** Representative figure of immunohistochemical localization of PEPT-2 in human ocular tissues. Arrowhead indicates the localization of PEPT-2 staining. PEPT-2 clearly localized in epithelial layers of cornea and conjunctiva and nonpigmented epithelial layer of ciliary body. In sclera-choroid-retina, PEPT-2 was abundantly localized in outer segment of photoreceptor cells, RPE cell layer and smooth muscles of...
choroid blood vessels. Light staining for PEPT-2 was also observed in ganglion cell layer. Nuclei were counterstained as blue with hematoxylin.

**Figure 5.** Representative figure of immunohistochemical localization of ATB\(^{0,+}\) in human ocular tissues. ATB\(^{0,+}\) showed the localization near nucleus. Arrowhead indicates the localization of ATB\(^{0,+}\) staining. ATB\(^{0,+}\) showed light staining in corneal and conjunctival epithelium and conjunctival stroma. ATB\(^{0,+}\) exhibited abundant expression in nonpigmented ciliary epithelium and RPE layer. Nuclei were counterstained as blue with hematoxylin.

**Figure 6.** Representative figure of immunohistochemical localization of OCT-1 in human ocular tissues. Arrowhead indicates the localization of OCT-1 staining. OCT-1 showed light staining in corneal epithelium, conjunctival epithelium, and nonpigmented ciliary epithelium. OCT-1 exhibited abundant expression in the inner segment of photoreceptor cells, RPE cell layer, and smooth muscles of choroidal blood vessels. Nuclei were counterstained as blue with hematoxylin.

**Figure 7.** Representative figure of immunohistochemical localization of OCT-2 in human ocular tissues. Arrowhead indicates the localization of OCT-2 staining. OCT-2 showed light expression in corneal and conjunctival epithelia. Nuclei were counterstained as blue with hematoxylin.

**Figure 8.** Representative figure of immunohistochemical localization of MCT-1 in human ocular tissues. Arrowhead indicates the localization of MCT-1 staining. MCT-1 showed light expression in corneal and conjunctival basal epithelial cells. MCT-1 showed strong labeling in nonpigmented ciliary epithelium compared to any other ocular tissues. For choroid- retina, MCT-1 staining was present in the outer segment of photoreceptor
cells, inner limiting membrane, and RPE cell layer. Nuclei were counterstained as blue with hematoxylin.

**Figure 9.** Representative image for immunohistochemical localization of MCT-3 transporter in human ocular tissues. MCT-3 immunolabeling was observed only in RPE layer, all other ocular tissues were devoid of labeling with MCT-3 antibody. Nuclei were counterstained as blue with hematoxylin.

**Figure 10.** Sclera-to-retina transport of Gly-Sar, MPP+, and L-tryptophan was higher than retina-to-sclera transport and significantly inhibited in the presence of the inhibitor cocktail, whereas there was no effect of inhibitors on the transport of phenylacetic acid. Effect of inhibitors on transport of A) Gly-Sar, B) MPP+, C) L-tryptophan, and D) Phenylacetic acid across human sclera-choroid-RPE. Data are expressed as mean ± SD for n =4 for sclera to retina direction. For retina to sclera direction, data are expressed as mean for n =2. * P ≤ 0.05 when compared with transport in the presence of inhibitor.

**Figure 11.** Transport of Gly-Sar, MPP+, L-tryptophan, and phenylacetic acid across human cornea is inhibited in the presence of an inhibitor cocktail. Effect of inhibitors on transport of A) Gly-Sar, B) MPP+, C) L-tryptophan, and D) Phenylacetic acid across human cornea. Data are expressed as mean ± SD for n =4 for apical to basal direction and mean of n= 2 for apical to basal direction with inhibitors.
Table 1: Patient demographic information.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Experiment performed</th>
<th>Sex</th>
<th>Age</th>
<th>Race</th>
<th>Lens Status</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>Transport</td>
<td>Male</td>
<td>69</td>
<td>Caucasian</td>
<td>Phakic</td>
<td>Renal Disease</td>
</tr>
<tr>
<td>02</td>
<td>Transport</td>
<td>Female</td>
<td>56</td>
<td>Caucasian</td>
<td>Phakic</td>
<td>Cerebrovascular Accident</td>
</tr>
<tr>
<td>03</td>
<td>Transport</td>
<td>Male</td>
<td>65</td>
<td>Caucasian</td>
<td>Phakic</td>
<td>Myocardial Infraction</td>
</tr>
<tr>
<td>04</td>
<td>Transport</td>
<td>Male</td>
<td>83</td>
<td>Caucasian</td>
<td>Aphakic</td>
<td>Renal Failure</td>
</tr>
<tr>
<td>06</td>
<td>IHC</td>
<td>Female</td>
<td>52</td>
<td>Caucasian</td>
<td>Phakic</td>
<td>Not known</td>
</tr>
<tr>
<td>07</td>
<td>IHC</td>
<td>Male</td>
<td>62</td>
<td>Caucasian</td>
<td>Phakic</td>
<td>Diabetes</td>
</tr>
<tr>
<td>08</td>
<td>IHC</td>
<td>Male</td>
<td>64</td>
<td>Caucasian</td>
<td>Aphakic</td>
<td>Heart Attack</td>
</tr>
</tbody>
</table>
Table 2: Summary of antibodies and conditions for immunohistochemistry of drug transporter in human ocular tissues.

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Primary Antibody</th>
<th>Dilution</th>
<th>Incubation Condition</th>
<th>Secondary Antibody</th>
<th>Antigen Retrieval Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEPT-1</td>
<td>Antihuman goat PEPT-1 Ab (Santa Cruz Biotech, Santa Cruz, CA)</td>
<td>1:200</td>
<td>60 min at 37 °C</td>
<td>Poly-AP antigoat IgG</td>
<td>High pH heat induced antigen retrieval (10 mM Tris-HCl, 1mM EDTA, pH 9.0 at 95 °C for 20 min)</td>
</tr>
<tr>
<td>PEPT-2</td>
<td>Antihuman rabbit PEPT-2 Ab (Santa Cruz Biotech, Santa Cruz, CA)</td>
<td>1:200</td>
<td>Overnight at 4 °C</td>
<td>Poly-AP antirabbit IgG</td>
<td>Low pH heat induced antigen retrieval (10 mM citrate buffer, pH 6.0 at 95 °C for 20 min)</td>
</tr>
<tr>
<td>OCT-1</td>
<td>Antihuman rabbit OCT-1 Ab (Santa Cruz Biotech, Santa Cruz, CA)</td>
<td>1:200</td>
<td>Overnight at 4 °C</td>
<td>Poly-AP antirabbit IgG</td>
<td>High pH heat induced antigen retrieval (10 mM Tris-HCl, 1mM EDTA, pH 9.0 at 95 °C for 20 min)</td>
</tr>
<tr>
<td>OCT-2</td>
<td>Antihuman rabbit OCT-2 Ab (Santa Cruz Biotech, Santa Cruz, CA)</td>
<td>1:200</td>
<td>60 min at 37 °C</td>
<td>Poly-AP antirabbit IgG</td>
<td>High pH heat induced antigen retrieval (10 mM Tris-HCl, 1mM EDTA, pH 9.0 at 95 °C for 20 min)</td>
</tr>
<tr>
<td>ATB^{0+}</td>
<td>Antihuman rabbit ATB^{0+} Ab (Medical and Biological Laboratories, Japan)</td>
<td>1:5000</td>
<td>60 min at 37 °C</td>
<td>Poly-AP antirabbit IgG</td>
<td>High pH heat induced antigen retrieval (10 mM Tris-HCl, 1mM EDTA, pH 9.0 at 95 °C for 20 min)</td>
</tr>
<tr>
<td>MCT-1</td>
<td>Antihuman goat MCT1 Ab (Santa Cruz Biotech, Santa Cruz, CA)</td>
<td>1:200</td>
<td>60 min at 37 °C</td>
<td>Poly-AP antigoat IgG</td>
<td>Low pH heat induced antigen retrieval (10 mM citrate buffer, pH 6.0 at 95 °C for 20 min)</td>
</tr>
<tr>
<td>MCT-3</td>
<td>Antihuman rabbit MCT3 Ab (Santa Cruz Biotech, Santa Cruz, CA)</td>
<td>1:200</td>
<td>60 min at 37°C</td>
<td>Poly-AP antirabbit IgG</td>
<td>No antigen retrieval</td>
</tr>
</tbody>
</table>
Table 3: List of transporters, specific substrates, and inhibitors for particular transporter and inhibition mechanism.

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Specific Substrate</th>
<th>Specific Inhibitor</th>
<th>Inhibition Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEPT</td>
<td>Gly-Sar</td>
<td>H-Pro-Phe-OH</td>
<td>Competitive Inhibition</td>
</tr>
<tr>
<td>OCT</td>
<td>MPP⁺</td>
<td>Metformin</td>
<td>Competitive Inhibition</td>
</tr>
<tr>
<td>ATB⁹⁺</td>
<td>L-Tryptophan</td>
<td>α-Methyl Tryptophan</td>
<td>Specific Inhibition (not a substrate)</td>
</tr>
<tr>
<td>MCT</td>
<td>Phenyl Acetic Acid</td>
<td>Nicotinic acid</td>
<td>Competitive Inhibition</td>
</tr>
</tbody>
</table>
**Table 4**: Summary of immunohistochemical localization of drug transporters in human ocular tissues. (+) indicates the immunostaining based presence and (-) indicates immunostaining based absence of transporter expression.

<table>
<thead>
<tr>
<th>Ocular Tissue</th>
<th>PEPT</th>
<th>OCT</th>
<th>MCT</th>
<th>ATB&lt;sup&gt;0,+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PEPT-1 PEPT-2</td>
<td>OCT-1 OCT-2</td>
<td>MCT-1 MCT-3</td>
<td></td>
</tr>
<tr>
<td>Cornea</td>
<td>+ +</td>
<td>+ +</td>
<td>+ -</td>
<td>+</td>
</tr>
<tr>
<td>Conjunctiva</td>
<td>+ +</td>
<td>+ +</td>
<td>+ -</td>
<td>+</td>
</tr>
<tr>
<td>Ciliary Epithelium</td>
<td>+ +</td>
<td>+ -</td>
<td>- - -</td>
<td></td>
</tr>
<tr>
<td>Choroidal smooth muscle</td>
<td>+ +</td>
<td>+ -</td>
<td>- - -</td>
<td></td>
</tr>
<tr>
<td>Retinal pigmented epithelum (RPE)</td>
<td>+ +</td>
<td>+ -</td>
<td>- - +</td>
<td>-</td>
</tr>
<tr>
<td>Outer segment of photoreceptor cells</td>
<td>- +</td>
<td>- -</td>
<td>+ - -</td>
<td></td>
</tr>
<tr>
<td>Inner segment of Photoreceptor cells</td>
<td>- +</td>
<td>+ -</td>
<td>- - -</td>
<td></td>
</tr>
<tr>
<td>Outer nuclear layer</td>
<td>- -</td>
<td>- -</td>
<td>- - +</td>
<td>+</td>
</tr>
<tr>
<td>Inner nuclear layer</td>
<td>- -</td>
<td>- -</td>
<td>- - +</td>
<td>+</td>
</tr>
<tr>
<td>Outer plexiform layer</td>
<td>+ -</td>
<td>- -</td>
<td>- - -</td>
<td></td>
</tr>
<tr>
<td>Inner plexiform layer</td>
<td>- -</td>
<td>- -</td>
<td>- - -</td>
<td></td>
</tr>
<tr>
<td>Ganglion cell layer</td>
<td>+ +</td>
<td>- -</td>
<td>- - +</td>
<td>+</td>
</tr>
<tr>
<td>Inner limiting membrane</td>
<td>- -</td>
<td>- -</td>
<td>+ - -</td>
<td></td>
</tr>
</tbody>
</table>
Figure 6
Cornea

OCT-2

Conjunctiva

Ciliary Body

Sclera-Choroid-Retina

Figure 7
Figure 10

**SCRPE Transport**

A) Gly-Sar (PEPT)
- Sclera → Retina
- Sclera → Retina (With Inhibitor)
- Retina → Sclera

B) MPP⁺ (OCT)
- Sclera → Retina
- Sclera → Retina (With Inhibitor)
- Retina → Sclera

C) L-Tryptophan (ATB⁺⁺⁺)
- Sclera → Retina
- Sclera → Retina (With Inhibitor)
- Retina → Sclera

D) Phenyl acetic acid (MCT)
- Sclera → Retina
- Sclera → Retina (With Inhibitor)
- Retina → Sclera

Cumulative % Transport vs. Time (Hr)
Cornea Transport

A) Gly-Sar (PEPT)
- Apical → Basal
- Apical → Basal (With Inhibitor)

B) MPP⁺ (OCT)
- Apical → Basal
- Apical → Basal (With Inhibitor)

C) L-Tryptophan (ATB⁰⁺)
- Apical → Basal
- Apical → Basal (With Inhibitor)

D) Phenyl acetic acid (MCT)
- Apical → Basal
- Apical → Basal (With Inhibitor)

Figure 11