Membrane Transporters as Targets for the Development of Drugs and Therapeutic Strategies

Yoshiyuki Kubo,* Shin-ichi Akanuma, and Ken-ichi Hosoya
Department of Pharmaceutics, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama; 2630 Sugitani, Toyama 930–0194, Japan.
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The retina is a tissue essential for vision, and the blood–retina barrier (BRB) helps to maintain an optimal microenvironment for the neural system in the retina. Recent findings concerning the BRB showed the involvement of transporters at the inner and outer BRB in drug and nutrient transport, suggesting their utility in the development of novel drug delivery systems to the retina. An in vitro–in vivo relationship study of permeability suggested the influx transport of verapamil, a cationic drug, across the BRB, and further in vivo and in vitro studies of cationic drugs, such as verapamil, propranolol and clonidine, revealed the involvement of carrier-mediated process in their influx transport at the BRB. Studies on substrate specificity in TR-1BRB2 cells, an in vitro model cell line of the inner BRB, suggests the involvement of novel organic cation transporter in the influx transport of cationic drugs at the inner BRB. Considering the neuroprotective effect previously reported for several cationic drugs, such as propranolol and clonidine, the study of cation transport at the BRB is widely expected to improve the treatment of retinal diseases, such as diabetic retinopathy and age-related macular degeneration.

Key words cation transport; membrane transporter; blood–retinal barrier; diabetic retinopathy; age-related macular degeneration; neuroprotectant

1. INTRODUCTION

The retina is an essential neural tissue involved in vision: visual information is processed by the retinal–neural system formed by the neural cells, such as rod cells, cone cells, bipolar cells, ganglion cells, horizontal cells and amacrine cells, and by Müller cells that are the retinal glial cells (Fig. 1). For the normal function of the retinal–neural system, the retinal microenvironment is required to be optimally sustained, suggesting the physiological importance of the blood–retinal barrier (BRB) in regulating material transport between the retina and circulating blood.1,2 In the retina, retinal capillary endothelial cells and retinal pigment epithelial (RPE) cells are responsible for the inner and outer BRB, respectively. The tight junction formed by these cells strictly limits non-specific material transport through the paracellular route, suggesting that transcellular transport significantly contributes to the supply of essential nutrients and the elimination of endobiotics and xenobiotics.2,3

In recent years, remarkable progress has been made in the study of transport at the BRB. The transcellular transport of various compounds at the BRB has been shown to involve carrier-mediated transport process, suggesting the major contribution of transporter molecules in the retinal capillary endothelial cells and RPE cells. As typical examples, studies of the inner BRB have suggested the role of transporters, such as glucose transporter (GLUT1/solute carrier 2A1 (SLC2A1)), L-type amino acid transporter (LAT1/SLC7A5), cationic amino acid transporter 1 (CAT1/SLC7A1), Na+-dependent multivitamin transporter (SMVT/SLC5A6), equilibrative nucleoside transporter 2 (ENT2/SLC29A2) and taurine transporter (TauT/SLC6A6), in supplying nutrients into the retina.4–10 Other studies have shown the expression of ATP-biding cassette (ABC) transporters, such as P-glycoprotein (P-gp/MDR1/ABCB1), multidrug resistance associated protein 4 (MRP4/ABCC4) and breast cancer resistance protein (BCRP/ABCG2), and suggest their contribution to the elimination of metabolites and xenobiotics.11–13

These research achievements suggest that various transporters contribute to maintaining a suitable retinal microenvironment for the retinal–neural system. Investigation of facilitative material transport at the BRB is widely expected to improve the treatment of such retinal diseases as diabetic retinopathy and age-related macular degeneration, since carrier-mediated transport at the BRB appears to be highly promising in the development of efficient and safe drug delivery systems to the retina.

2. IN VITRO–IN VIVO RELATIONSHIP IN TRANSPORT ACROSS THE BRB

In the study of material transport across the BRB, the retinal uptake index (RUI) has been viewed as a useful in vivo method to evaluate the permeability of compounds at the BRB.14 RUI is available from a calculation based on data experimentally obtained from carotid artery injection

* To whom correspondence should be addressed. e-mail: kuboyoshi@pha.u-toyama.ac.jp

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of compounds with a diffusible internal reference, such as $[^{14}C]n$-butanol; RUI expresses the fractional uptake of the test compound in the retina as a percentage of the fractional uptake of the diffusible internal reference compound. However, RUI is considered to be an adverse method in the investigation of multiple samples; we need to establish a quick and easy in vitro method for the high throughput screening conducted in industrial drug discovery, clearly suggesting the need to clarify the in vitro–in vivo relationship of membrane permeability at the BRB.

To date, permeability studies involving RUI and in vitro cell uptake have shown that TR-iBRB2 cells, an in vitro model cell line of the inner BRB, are useful in evaluating the permeability of drugs and nutrients at the BRB. The latest results show a correlation between the initial uptake rate ($\log F$) obtained in TR-iBRB2 cells and RUI of compounds undergoing carrier-mediated transport and passive diffusion, respectively, and exhibited trend lines: Eq. 1 ($RUI=26.5 \times \exp(0.887 \times \log F)$) and Eq. 2 ($RUI=26.5 \times \exp(1.55 \times \log F)$) (Figs. 2A, B), suggesting the great utility of TR-iBRB2 cells in drug discovery for treating the retinal diseases.

In an early verification study of the in vitro–in vivo relationship, test compounds were classified into two groups: compounds undergoing passive diffusion (Group I), and carrier-mediated transport (Group II) (Table 1). Group I exhibited a lipophilicity trend line (Eq. 3; $RUI=46.2 \times \exp(0.515 \times \log DC)$) derived from both lipophilicity ($\log DC$) and in vivo permeability ($RUI$) (Fig. 2C). Similarly, in the study of Group I at the BBB, a lipophilicity trend line (Eq. 4; $BU1=24.2 \times \exp(0.816 \times \log DC)$) was also shown for the $\log DC$ and the brain uptake index (BUI) that expresses the fractional uptake of the test compound in the brain, as with the $RUI$ (Fig. 2D). Regarding Group II, the measured RUI values of $\alpha$-glucose, $\alpha$-arginine, $\alpha$-leucine, $\alpha$-phenylalanine and biotin were shown to be greater than the values predicted by means of Eq. 3, and similar results were also observed for the BUI (Fig. 2C, D), suggesting the involvement of transporters, such as GLUT1, CAT1, LAT1 and SMVT, expressed at the BRB and BBB in the influx transport of nutrients into the retina and brain (Fig. 3A). In addition, the involvement of LAT1 was suggested in the influx transport of $\alpha$-dopa for the treatment of Parkinson’s disease across both the BRB and BBB, since $\alpha$-dopa is a derivative of amino acid and its measured RUI and BUI was greater than predicted (Fig. 2C, D).

Verapamil is a cationic drug, and its measured RUI value was also much greater than predicted, although its measured BUI was comparable to that predicted, suggesting a marked difference of barrier function between the BRB and BBB. Verapamil, vincristine and digoxin are known as the typical drug substrates of P-gp, of which expression at the BRB was previously reported; their function at the BRB is clearly
supported by the results in which vincristine and digoxin exhibited lower RUI values than those predicted (Fig. 2C). These results show that the BRB and BBB function differently for verapamil, suggesting the facilitative influx transport of verapamil at the BRB (Fig. 3B).

3. CATIONIC DRUG TRANSPORT AT THE BRB

Several cationic drugs, such as memantine, propranolol and clonidine, have recently been reported to exert a neuroprotective effect in the brain and retina, and these reports support the substantial contribution of the study of cationic drug transport at the BRB in developing future treatments for retinal disease. In the in vivo transport study of verapamil in rats, the apparent influx clearance of [3H]verapamil ($K_{in, verapamil, retina}$) was calculated to be 614 µL/(min g retina), which was much greater than that of paracellular transport markers, supporting the facilitative influx transport system.
of verapamil at the BRB. In the in vivo inhibition study of $[^3H]$verapamil transport, the RUI was significantly reduced in the presence of pyrilamine, a cationic drug, whereas the BUI was increased by pyrilamine.21 In the further assessment of differences in the BRB and BBB, a study with $P$-gp knockout rats showed the negligible impact of $P$-gp on $K_{in,verapamil,retina}$ in spite of a marked alteration of the $K_{in,verapamil,brain}$. Similar results were reported in a study using $P$-gp/Bcrp knockout mice.23 These findings strongly support differences in transport properties of the BRB and BBB, and suggest the dominant influx transport of verapamil at the BRB (Fig. 3B).

Because of the neuroprotective effects of cationic drugs, as previously reported,18-20 the influx transport system of cationic drugs at the BRB is expected to be helpful in the delivery of neuroprotectants into the retina. Among such cationic neuroprotectants, propranolol reduces the expression of vascular endothelial growth factor (VEGF), and clonidine induces basic fibroblast growth factor (bFGF) to exert a neuroprotective effect.19,20 In the in vivo transport study, the measured RUI values of $[^3H]$propranolol and $[^3H]$clonidine were much greater than predicted values (Fig. 2C), and were inhibited in the presence of cationic drugs, such as verapamil and pyrilamine.24,25 These results suggest the involvement of facilitative transport system in the influx transport of propranolol and clonidine at the BRB, supporting the utility of these transport systems in the efficient and safe delivery of cationic neuroprotectants to the retina.

4. CARRIER-MEDIATED TRANSPORT OF CATIONIC DRUGS AT THE INNER BRB

Referring to the influx transport of cationic drugs including neuroprotectants at the BRB, detailed investigations will likely contribute to our understanding of drug distribution and drug–drug interaction in the retina. In the study with TR-iBRB2 cells, the uptake of $[^3H]$verapamil occurred in a time-, temperature- and concentration-dependent manner21 (Fig. 4A), and these results suggest the involvement of carrier-mediated process in the influx transport of verapamil at the inner BRB. Similarly, in the in vitro uptake study of $[^3H]$propranolol and $[^3H]$clonidine, these drugs also revealed time-, temperature- and concentration-dependent transport properties, suggesting the involvement of carrier-mediated process in the influx transport of cationic neuroprotectants into the retina.

In the in vitro inhibition study, the substrate specificity of cationic drug transport at the inner BRB was investigated. In TR-iBRB2 cells, the uptake of $[^3H]$verapamil was significantly inhibited by cationic drugs, including quinidine, pyrilamine, mecamylamine, amantadine, propranolol and clonidine, whereas no significant effect was shown by $p$-aminohippuric acid (PAH), a typical anionic compound (Fig. 4C), suggesting the involvement of organic cation transporter in verapamil transport at the inner BRB.21 Similar results were obtained for $[^3H]$propranolol and $[^3H]$clonidine, suggesting that the transports of propranolol and clonidine also involve organic cation transporter at the inner BRB. Furthermore, the uptakes of $[^3H]$verapamil, $[^3H]$propranolol, $[^3H]$clonidine and $[^3H]$pyrilamine were significantly inhibited by cationic neuroprotectants, such as desipramine and memantine,21,24,25 strongly suggesting that the delivery of neuroprotectants through carrier-mediated cationic drug transport is a promising way to improve the treatment of retinal diseases.

Based on functional properties, including substrate specificity and ion-sensitivity, the cationic drug transport at the BRB is assumed to involve a number of organic cation transporters (Fig. 5). The in vitro inhibition study revealed that substrate specificity of the cationic drug transport at the BRB is different from those of well-characterized organic cation transporters, such as plasma membrane monoamine transporter (PMAT/SLC29A4/ENT4), multidrug and toxin extrusion (SLC47/A1), organic cation/carnitine transporters (OCTNs/SLC22A4–5), and organic cation transporters (SLC22A1–3).21,24,25 suggesting the involvement of novel organic cation transporters in the influx transport of cationic drugs across the BRB.

5. CONCLUSION

In 1913, Schnaudigel proposed the barrier structure between
the circulating blood and retina, and a century-old study has been demonstrated for the BRB. From the 1960s onward, the BRB was thought to be a static barrier to limit non-specific material transport between the blood and retina, based on the tight junction formed by the responsible cells, such as capillary endothelial cells and RPE cells. However, because the retina is a small neural tissue, it has been difficult to assess in detail material transport in the retina. From the 2000s onward, TR-iBRB2 cells, an in vitro model cell line, were established as useful in in vitro transport studies involving the BRB. 

**Fig. 4. In Vitro Study of Cationic Drug Transport at the Inner BRB**

The uptake study of [3H]verapamil (A) and [3H]clonidine (B) was performed by means of TR-iBRB2 cells, an in vitro model cell line of the inner BRB, and the concentration-dependent transports suggest the involvement of carrier-mediated transport process in the influx transport of verapamil and clonidine across the inner BRB. The substrate specificity of [3H]verapamil transport was examined in TR-iBRB2 cells, and the results suggest the involvement of novel organic cation transporters in the influx transport of cationic drug across the inner BRB. The figure was prepared by reference to Kubo Y, Kasagawa Y, Tachikawa M, Akanuma S, Hosoya K, “Involvement of a novel organic cation transporter in verapamil transport across the inner blood–retinal barrier,” 30, 847–856 (2013) with permission from Springer; and to Kubo Y, Tsuchiyama A, Shimizu Y, Akanuma S, Hosoya K, “Involvement of carrier-mediated transport in the retinal uptake of clonidine at the inner blood–retinal barrier,” 11, 3747–3753, (2014) with permission from ACS Publications. PAH, p-aminohippuric acid.

**Table 2. Kinetic Parameters Suggested for Cationic Drug Transports at the Inner BRB**

| Drugs   | $K_m$ (µM) | $V_{max}$ (nmol/(min·mg protein)) | $V_{max}/K_m$ (µL/(min·mg protein)) |
|---------|------------|----------------------------------|-------------------------------------|
| Verapamil | 61.9       | 3.31                             | 53.5                                |
| Pyrilamine | High affinity | 20.2                             | 0.837                               | 41.4                                |
|          | Low affinity | 252                              | 22.3                                | 88.5                                |
| Propranolol | 237       | 11.9                             | 50.2                                |
| Clonidine | 287        | 43.7                             | 152                                |

This table was prepared by reference to previous reports where kinetic parameters were estimated in the in vitro uptake study in TR-iBRB2 cells.
BRB. Therefore, further detailed study of the function and expression of transporters at the BRB is expected to make a large contribution toward improving the treatment of retinal diseases.

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Conflict of Interest The authors declare no conflict of interest.

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