DDT Vol. 6, No. 4 February 2001

Efflux transport systems for drugs at the blood–brain barrier and blood–cerebrospinal fluid barrier (Part 2)

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Penetration of the blood–brain barrier or blood–cerebrospinal fluid barrier is necessary if a drug is to achieve the required concentration for a desired pharmacological effect. Efflux transport systems at such barriers provide protection for the CNS by removing drugs from the brain or cerebrospinal fluid, and transferring them to the systemic circulation. In Part 2 of this review, *in vivo* and *in vitro* studies of efflux transport via these barriers are discussed, with reference to the transporters previously described in Part 1[1].

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▼ Efflux transport of organic cations and neutral compounds across the BBB

Two studies[2,3] have investigated the mechanism that restricts the brain penetration of doxorubicin and cyclosporin A by using rats with transient brain ischemia, caused by occlusion of the vertebral and common carotid arteries. The brain uptake clearance of cyclosporin A and doxorubicin determined by an *in situ* brain perfusion technique demonstrated a threefold (from 100 to 315 μl min^{-1}) and a 17-fold increase (from 14 to 243 μl min^{-1}) under such conditions, respectively[2,3]. There was no significant difference in the brain uptake clearance of [^{14}C]sucrose after occlusion compared with that in rats without the occlusion. This suggests that the increase in the brain uptake clearance of doxorubicin and cyclosporin A caused by brain ischemia cannot be attributed to an increase in non-specific transport via the paracellular route[2,3]. This was further supported by the observation that the brain uptake clearance of doxorubicin returned to normal after 30 min and 24 h of cerebral recirculation of blood following transient ischemia[2]. ATP concentration in the brain, measured 20 min after occlusion, was reduced to 3% of the value in rats without occlusion, and returned to normal after the recirculation of blood[2]. The brain uptake clearance of doxorubicin was inversely correlated with the ATP concentration in the brain, which was confirmed *in vitro* using primary cultured bovine brain capillary endothelial cells (BCECs)[2]. These results suggest that the mechanism restricting the brain uptake of these drugs is located at the blood–brain barrier (BBB) and is driven by ATP.

Some drugs can also increase the brain uptake of other drugs from the blood. For example, when quinidine was administered via a microdialysis probe implanted into rat brain, the brain uptake of cyclosporin A increased twofold[3] during single-pass after bolus-input to the carotid artery (brain uptake index method[4]). Furthermore, verapamil, cyclosporin A and progesterone increase the intracellular accumulation of vincristine in bovine and rat primary cultured BCEC[5,6]. Correspondingly, verapamil inhibits the efflux of vincristine from the primary cultured BCEC[7]. Two studies used immortalized mouse BCEC (known as MBEC4) cultured on a porous membrane, and measured the transcellular transport of vincristine and cyclosporin A (Refs 7,8). Basal–apical transport of these compounds was greater compared with transport from the opposite direction and was reduced in the presence of verapamil[7,8]. This *in vitro* transport might correspond to *in vivo* brain–blood

1359-6446/01/$ – see front matter PII: S1359-6446(00)01643-3

Table 1. $K_{p,\ brain}$ values in Mdr1a(–/–) and Mdr1a(+/+) mice after intravenous injection of various drugs

Drugs	$K_{p,brain}$		Ratio ($K_{p,brain,ko}/K_{p,brain,wt}$)	Time (h) after administration	Ref.
	Mdr1a(–/–)	Mdr1a(+/+)			
Asimadoline[a]	2.9	0.31	9.4	1	39
Cyclosporin A	3.3	0.3	11.0	4	40
Dexamethasone	0.7	0.3	2.3	4	40
Digoxin	1.5	0.08	18.8	4	40
Doxorubicin	0.0025	0.00077	3.2	1	41
FK506[b]	16.4	2.73	6.0	5	42
Grepafloxacin[b,c]	1	0.34	2.9	2	43
HSR903	0.96	0.38	2.5	2	16
Indinavir	17.0	1.6	10.6	4	44
Ivermectin	0.59	0.041	14.4	4	45
Loperamide	2.0	0.3	6.7	4	18
Morphine	0.72	0.47	1.5	4	40
Ondansetron	1.9	0.5	3.8	0.5	18
Quinidine	4.8	0.2	24.0	0.16	15
SDZ PSC833	1.2	0.6	2.0	4	46
Sparfloxacin[b,c]	0.54	0.14	3.9	2	43
Verapamil	3.3	0.4	8.3	1	17
Vinblastine	18.7	1.7	11.0	4	10
Colchicine[d]	1	0.26	3.8		13

Drugs were administered intravenously to both wild-type and Mdr1a or Mdr1a/Mdr1b double knockout mice. Plasma and brain concentrations were determined at the time of death. $K_{p,brain}$ was obtained by dividing the amount associated with the brain by the plasma concentration.
[a]$K_{p,brain}$ of asimadoline is the tissue:blood concentration ratio.
[b]$K_{p,brain}$ values of grepafloxacin and sparflocaxin were evaluated using plasma unbound concentration.
[c]Mdr1a/Mdr1b double knockout mice were used for this experiment.
[d]The brain uptake clearance of colchicine was determined using an *in situ* brain perfusion technique.

transport, if the apical localization of P-glycoprotein on MBEC4 cells is taken into consideration[1,7]. These results suggest that an active efflux transport system at the BBB is responsible for extruding drugs into the systemic circulation.

The drugs described in this section are either substrates or inhibitors of P-gp, and the anti-human P-gp neutralizing antibody, MRK-16, increases the intracellular accumulation of cyclosporin A in primary cultured bovine BCECs[5]. Taken together, these facts suggest that P-gp plays a role in BBB function.

Studies using Mdr1a and Mdr1a/Mdr1b knockout mice

The Mdr1a and Mdr1b isoforms of P-gp are primary active transporters with broad substrate specificity that confer multidrug resistance to cells, as discussed in Part 1 of this review[1]. Mdr1a and Mdr1a/Mdr1b double knockout mice have been generated by Schinkel and coworkers[9,10]. In addition, Umbenhauer and colleagues have found naturally occurring subpopulations of CF-1 mice that are Mdr1a-deficient[11]. No expression of P-gp in the BBB and the small intestine was observed in Mdr1a knockout mice[10,12], but the expression of Mdr1b in the liver and kidney was found to be increased[10]. Furthermore, Mdr1a knockout mice and Mdr1a-deficient CF-1 mice are much more sensitive (3–100-fold) than normal mice to the neurotoxicity caused by avermectins, such as abamectin and ivermectin, and to vinblastine[10,12]. The brain uptake of ivermectin and vinblastine is markedly increased 26-fold and 11-fold, respectively, in Mdr1a knockout mice[10,12].

Two studies have confirmed that the integrity of the BBB is maintained in Mdr1a knockout mice and Mdr1a-deficient CF-1 mice by comparing the brain–plasma partition coefficient ($K_{p,\ brain}$) of fluorescein and fluorescein-dextran-4000 with the vascular volume of sucrose and inulin, respectively[13,14]. The brain concentration of P-gp substrates was increased significantly in knockout mice compared with that in normal mice (Table 1)[10,13,15–18]. Although the brain uptake was expressed as $K_{p,\ brain}$, a significant difference was still observed (Table 1).

To examine the role of P-gp at the BBB, the alteration of the plasma concentration–time profile should be considered. Prolonged plasma elimination, caused by a lack of

P-gp-mediated biliary and urinary excretion, might contribute, at least in part, to the increase in brain concentration. Dagenais and coworkers evaluated the brain uptake clearance of colchicine using an *in situ* brain perfusion technique, and found that it increased by two- to four-fold in Mdr1a-deficient CF-1 mice compared with normal mice[13]. These results further suggest that P-gp is involved in one of the barrier transport mechanisms at the BBB.

Efflux transport of organic anions across the BBB

In vivo *studies using an intracerebral microinjection technique*

As described previously[1], two studies have found that the efflux of 1-napthyl-17β-glucuronide (N17βG), and the cyclic peptide RC160, following injection into the cerebral cortex, is carrier mediated[19,20]. Using the brain efflux index (BEI) technique, it was demonstrated that the efflux transport of *p*-aminohippurate (PAH), azidodeoxythymidine (AZT), dideoxyinosine (DDI), taurocholate (TCA) and the endothelin antagonist BQ123 is also carrier-mediated[21–23]. Although AZT and DDI are nucleoside analogues and are not negatively charged, their efflux from the brain is thought to be via the same efflux transport system as for organic anions[22]. After injection, these drugs are eliminated from the brain with a rate constant value of 0.059 (PAH), 0.032 (AZT), 0.025 (DDI), 0.023 (TCA) and 0.078 (BQ123) min^{-1}, respectively[21–23]. The saturable component of the barrier accounted for a major proportion of the total efflux of TCA and PAH from the brain, and 50%–60% of the total efflux of BQ123 and DDI (Refs 21–23). The apparent K_m values of PAH, TCA, BQ123 and DDI, which were determined using the concentration of non-radiolabeled ligand in the injectate, were 2.4, 0.4 and 2.9 nmol/0.2 µl injectate and 0.8 nmol/0.5 µl injectate, respectively[21–23]. The quantity of residual AZT radioactivity in the brain was increased only slightly in the presence of a high substrate concentration and organic anions, such as probenecid, PAH and benzylpenicillin, which suggests that transporters for organic anions might make only a minor contribution[22].

Because TCA and BQ123 inhibited each other's elimination from the brain following injection[23], it is possible that they are eliminated from the brain via the same mechanism. Furthermore, because the efflux of TCA via the BBB is not inhibited by PAH at 10 nmol/0.2 µl injectate, which is the concentration sufficient to saturate PAH efflux via the BBB, then the efflux transport system for TCA might differ to that for PAH[21,23]. These results suggest the existence of at least two efflux transport mechanisms at the BBB for TCA and BQ123, and for PAH. The organic anion transporter polypeptide 2 (Oatp2) is expressed at the plasma membrane of isolated rat brain capillary, and, because Oatp2 accepts TCA and BQ123 as substrates[24], it is a possible transporter involved in the efflux of these compounds from the brain[1].

Primary active transport in immortalized BCECs

A primary active transporter that restricts brain uptake of organic anions at the BBB has not yet been identified. In a series of studies carried out using MBEC4 cells[25,26], the uptake of glutathione (GSH) conjugates, such as dinitrophenyl–*S*-glutathione (DNP–*S*G) and leukotriene C_4 (LTC_4), into membrane vesicles was stimulated by ATP, but not by ADP or AMP[25]. Reduction of the ATP-dependent uptake of DNP–SG caused by an ATPase inhibitor, vanadate, suggests the involvement of a primary active transporter[25].

Monochlorobimane (MCB) is an agent used to measure the intracellular content of reduced-GSH. MCB undergoes conjugation with GSH inside cells to form glutathione–bimane (GS–B), which is fluorescent. When MBEC4 cells are cultured on a porous membrane, GS–B is preferentially excreted into the apical side[26]. As the concentration of MCB in the medium increased, the excretion of GS–B into the basal side was significantly increased compared with the apical side[26]. This result can be accounted for by the difference in the affinity of GS–B for two different transporters, because kinetic analyses using membrane vesicles prepared from MBEC4 cells revealed that there are two binding sites for the ATP-dependent uptake of GS–B (Ref. 26). It has been suggested that these high- and low-affinity sites correspond to transporters on the apical and basal membrane, respectively, and that excretion into the apical side is saturated even at the lowest concentration of MCB. The expression of multidrug resistance-associated protein (Mrp1) has been confirmed in MBEC4 cells by RT-PCR and Western blot analysis[25], and it has been proposed that Mrp1 is responsible for the efflux transport of GSH conjugates on the apical side. The transporter on the basal side remains unidentified: as discussed in Part 1 of this review[1], MRP1 expression at the BBB is debatable.

Efflux transport via the blood–cerebrospinal fluid barrier

The blood–cerebrospinal fluid barrier (BCSFB) consists of choroid epithelial cells that are tightly connected to each other and polarized to form brush-border and basolateral membranes[27,28]. The brush-border membrane of choroid epithelial cells faces the cerebrospinal fluid (CSF) (Fig. 1). CSF fills the ventricle and bathes the brain, and is secreted from the choroid plexus and absorbed into the venous blood flow through the arachnoid membrane, which results in a physiological flow (bulk flow). In the rat, this

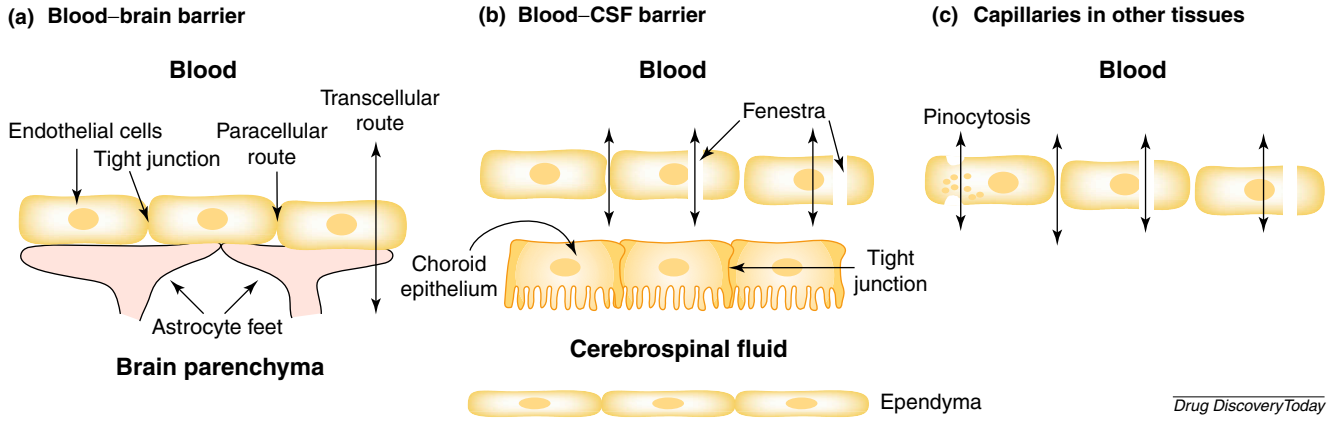

Figure 1. Schematic diagrams of the blood–brain barrier [BBB, **(a)**] and blood–cerebrospinal fluid barrier [BCSFB **(b)**]. The tight junction is a specific feature of brain capillary endothelial cells and choroid epithelial cells. It minimizes the penetration of drugs from circulating blood into the brain parenchyma and cerebrospinal fluid via the paracellular route. Therefore, drugs have to penetrate the CNS transcellularly. These barriers restrict the penetration of drugs that have low intrinsic permeability across the lipid bilayer because of their high MW and/or high hydrophilicity. This leads to poor drug distribution in the brain. Both brain capillary endothelial cells and choroid epithelial cells provide a barrier function to protect the CNS and are referred to as the blood–brain barrier and the blood–cerebrospinal fluid barrier, respectively. Transporters play an important role in restricting the penetration of drugs into the CNS by removing them into the circulating blood. **(c)** shows the mechanisms of transport that occur in the blood capillaries of other tissues where cell–cell junctions are not as tight as in the BBB and BCSFB. Abbreviation: CSF, cerebrospinal fluid.

bulk flow rate is 2.9 μl min^{-1}, therefore, the CSF (250 μl per rat) is replaced every 2–3 h. The efflux transport of organic anions from the CSF has been characterized *in vivo* by cerebroventricular–cisternal perfusion and intracerebroventricular administration[27,28]. In addition to bulk flow, there are two elimination routes from the CSF, via the choroid plexus and ependyma surface[27,28]. The elimination from the CSF via the choroid plexus consists of two steps: uptake from the CSF to the choroid plexus via the brush-border membrane, followed by excretion from the cell to the circulating blood through the basolateral membrane (Fig. 1).

Efflux transporters for organic anions on the choroid plexus

Organic anion transporting polypeptide 1 (Oatp1) is located on the brush-border membrane of the choroid plexus, whereas Oatp2 and Mrp1 are on the basolateral membrane in rat (Fig. 2)[1]. The elimination of estradiol-17β-glucuronide ($E_2$17βG, a typical substrate of Oatp1, Oatp2 and Mrp1) from the CSF is more rapid than the bulk flow rate after intracerebroventricular administration, and elimination can be inhibited by probenecid[29]. N17βG is also eliminated from the CSF and this efflux is saturated at high substrate concentration[19]. These organic anion transporters are considered to be responsible for the efflux of $E_2$17βG and N17βG from the CSF into the systemic circulation. Furthermore, because benzylpenicillin does not inhibit the uptake of $E_2$17βG into the isolated rat choroid plexus, the uptake mechanism of benzylpenicillin is thought to be different from that of $E_2$17βG (Ref. 29). In terms of driving force, the uptake mechanism of 2,4-dichlorophenoxyacetate (2,4D) is also thought to be different from that of $E_2$17βG (Ref. 30). Whether benzylpenicillin and 2,4D share the same uptake mechanism in the choroid plexus remains to be clarified, and, as yet, no information regarding their mechanism of excretion at the choroid plexus is available.

Studies using Mrp1 knockout mice

Studies using Mrp1 knockout mice could aid our understanding of the role of human MRP1 at the BCSFB. Sensitivity to etoposide is increased in Mrp1 knockout mice[31]. Because etoposide is a substrate of P-gp, Wijnholds and colleagues investigated the role of Mrp1 using Mdr1a/Mdr1b double knockout mice and Mrp1/Mdr1a/Mdr1b triple knockout mice[32]. CSF was collected during the experiment using a perfusion technique after intravenous administration of etoposide, and the plasma and brain concentrations of etoposide were determined at the time of death[32]. There was no significant difference in plasma and brain concentrations of etoposide between the double and triple knockout mice, suggesting a minor role for Mrp1 in barrier function at the BBB, at least where etoposide is concerned. However, the CSF concentration of etoposide in Mrp1/Mdr1a/Mdr1b triple knockout mice was significantly increased: eightfold relative to the CSF concentration in the double knockout mice and threefold

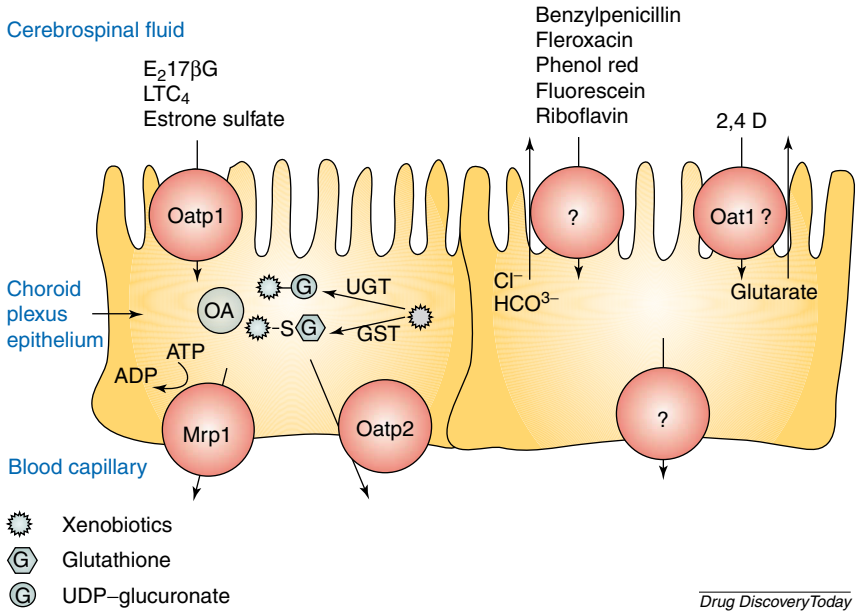

Figure 2. A possible scheme for efflux transporter systems for organic anions at the blood-cerebrospinal fluid barrier of rat is shown. Oatp1 and Oatp2 are expressed on the brush-border and basolateral membrane, respectively. Mrp1 expressed on the basolateral membrane is considered to actively eliminate glutathione conjugates and glucuronides that are formed in the choroid epithelial cells. They are considered to be responsible for the efflux transport of estradiol-17β-glucuronide ($E_2$17βG) and 1-naphthyl-17β-glucuronide. Although the molecular mechanisms have not yet been identified, the uptake systems for benzylpenicillin and 2,4 D are suggested to be different from Oatp1. Abbreviations: OA, organic anion; Oatp1, organic anion transporting polypeptide 1; Oatp2, organic anion transporting polypeptide 2; Oat 1, organic anion transporter 1; Mrp1, multidrug resistance associated protein 1; UGT, UDP-glucuronosyl transferase; GST, glutathione transferase; LTC_4, leukotriene C_4; 2,4 D, 2,4-dichlorophenoxyacetate.

relative to the brain concentration in the triple knockout mice. This can be accounted for by the reduction of efflux from CSF in the triple knockout mice. Further studies are required to quantitate the contribution of Mrp1 to the elimination of a substrate of Mrp1 from the CSF, comparing either the double and triple knockout mice, or wild-type and Mrp1 knockout mice.

Uptake mechanism of benzylpenicillin into the choroid plexus

In vivo studies using techniques such as cisternal-magna perfusion and intracerebroventricular administration have revealed that there is an efficient efflux transport system in the choroid plexus that removes 50% of benzylpenicillin during a single pass in the dog, and accounts for 64% of total efflux from the CSF in the rat[27]. The uptake of benzylpenicillin into the choroid plexus has been characterized *in vitro* using isolated rat choroid plexus[27]. The K_m value for the uptake of benzylpenicillin into the rat choroid plexus was determined to be 64 μM, which is consistent with a value determined previously *in vivo* (43 μM)[27]. A metabolic inhibitor, dinitrophenol, reduces the uptake of benzylpenicillin, which suggests the involvement of active transport[27]. An outward-concentration gradient was produced by preloading anions, such as Cl^-, HCO_3^-, SCN^- and SO_4^{2-}, into the ATP-depleted choroid plexus, and the uptake of benzylpenicillin was measured[27]. A stimulatory effect with preloaded Cl^- or HCO_3^-, but not with other anions, was observed for benzylpenicillin uptake into the choroid plexus. These anions are, therefore, candidates for the driving force behind benzylpenicillin uptake into the choroid plexus (Fig. 2)[27]. The substrate specificity of this uptake system has been examined kinetically by comparing K_m and K_i values. In addition to β-lactam antibiotics, cefodizime (a cephalosporin antibiotic), fleroxacin (a quinolone antibiotic), fluorescein, phenol red and riboflavin (Vitamin B_2) share the same efflux transport system as benzylpenicillin (Fig. 2)[33–35], although the transporter responsible for this has not yet been identified.

Uptake mechanism of 2,4D into the choroid plexus

The uptake of 2,4D into the isolated rabbit choroid plexus is saturable (K_m value = 72 μM) and inhibited by probenecid and salicylate[36]. These *in vitro* results are consistent with *in vivo* observations: 2,4D undergoes more rapid elimination from the CSF than inulin, and the elimination of 2,4D from CSF is inhibited by salicylate[36]. The inhibitory effect of the Na^+/K^+ uptake inhibitor, ouabain, suggests the partial involvement of a sodium-dependent uptake mechanism.

Pritchard and coworkers found that glutarate stimulated the uptake of 2,4D into rat isolated choroid plexus, and that this was dependent on extracellular sodium[30]. In addition, an outwardly directed gradient of glutarate across the membrane stimulated the initial uptake of 2,4D into brush-border membrane vesicles prepared from choroid plexus[30]. Because a sodium-dependent uptake system of glutarate is located on the brush-border membrane[30], the uptake mechanism of 2,4D is thought to be driven by glutarate, which is accumulated by the sodium-glutarate co-transporter (Fig. 2)[30]. This transport property is similar

to the organic anion transporter (OAT1)[1]. Although Pritchard and coworkers have demonstrated that an Oat1–green-fluorescent-protein (GFP) fusion protein is localized to the brush-border membrane of the choroid plexus[30], the expression of OAT1 in the choroid plexus has not been confirmed. Whether OAT1 accepts 2,4D as a substrate also remains to be elucidated.

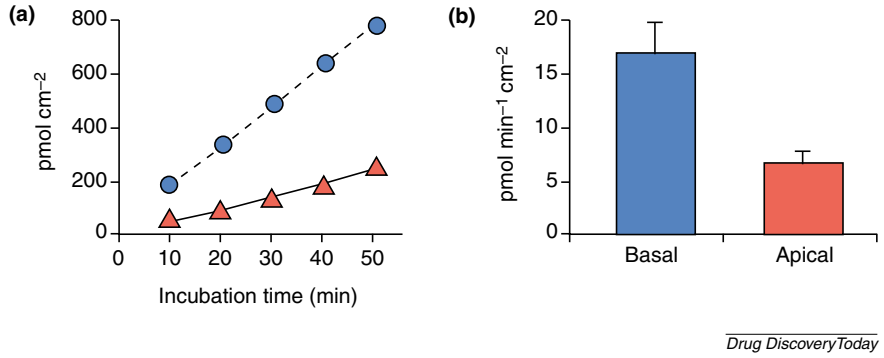

Figure 3. A monolayer of rat choroid epithelial cells was cultured on a porous membrane and incubated in the presence of 1-napthol. The excreted amount of 1-naphthol-17β-glucuronide (N17βG) was determined every 10 min. The time-profiles of the cumulative excretion (red, apical; blue, basal) of N17βG are shown in **(a)**. The excretion rate was obtained from the profile of the cumulative excretion amount of N17βG.
(b) N17βG formed in the choroid epithelial cells was preferentially excreted into the basal side. The excretion rate of N17βG into the basal side is ~threefold greater than that into the apical side. This suggests that UDP-glucuronosyl transferase and efflux transporters play a role in restricting the entry of xenobiotics into the cerebrospinal fluid. Figure reproduced, with permission, from Ref. 38.

Excretion of conjugated metabolites across the basolateral membrane of the choroid plexus

The enzymatic activity of glucuronidation (UDP-glucuronosyl transferase; UGT) per unit weight of tissue in the choroid plexus is as high as that in the liver[37]. Therefore, this suggests that xenobiotics undergo glucuronidation in the choroid plexus followed by efflux into the systemic circulation. Strazielle and coworkers evaluated the excretion of N17βG from primary cultured rat choroid epithelial cells by loading them with 1-naphthol[38]. The cumulative amount of N17βG excreted into the basal side was threefold greater than that into the apical side (Fig. 3)[38]. This difference corresponds to the difference in efflux transport activities. The excretion of N17βG into basal side medium was inhibited by probenecid, suggesting the involvement of a transporter[38]: Mrp1 and Oatp2 are thought to be responsible for this excretion.

Concluding remarks

The efflux transport processes at the BBB and BCSFB, which are major factors determining the brain concentration of drugs has been summarized. Kinetic analyses revealed that active efflux from the brain or CSF maintains drug concentrations in the CNS at a lower level than the unbound concentration in the blood. To date, a number of transporters have been identified. P-gp has been shown to restrict the penetration of drugs into the brain. Some transporters such as Oatp1, Oatp2 and MRP1 are thought to be involved in the elimination of drugs from the brain and CSF. Further studies are required to confirm whether these transporters are able to completely explain CNS drug disposition.

Gene expression systems of human transporters are useful tools for examining their transport properties *in vitro*. To extrapolate the results obtained from *in vitro* studies to *in vivo* quantitatively, the contribution of each transporter to the total efflux transport must be evaluated. Primary cultures and immortalized cell lines have served as *in vitro* models of the barriers. However, dedifferentiation of BCECs when they are cultured or immortalized is a major problem, which will influence the down- and up-regulation of transporters, transport via the paracellular route and transendothelial electrical resistance. Further studies are required to confirm whether these *in vitro* models maintain expression of transporters, and, hence, are valuable in the study of barrier function.

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