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Advanced Drug Delivery Reviews 36 (1999) 179–194

advanced drug delivery reviews

P-Glycoprotein, a gatekeeper in the blood–brain barrier

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Abstract

The blood–brain barrier is a major impediment to the entry of many therapeutic drugs into the brain. P-Glycoprotein is an ATP-dependent drug transport protein that is predominantly found in the apical membranes of a number of epithelial cell types in the body, including the blood luminal membrane of the brain capillary endothelial cells that make up the blood–brain barrier. Since P-glycoprotein can actively transport a huge variety of hydrophobic amphipathic drugs out of the cell, it was hypothesized that it might be responsible for the very poor penetration of many relatively large (>400 Da) hydrophobic drugs in the brain, by performing active back-transport of these drugs to the blood. Extensive experiments with in vitro models and with knockout mice lacking blood–brain barrier P-glycoprotein or other animal models treated with blockers of P-glycoprotein have fully confirmed this hypothesis. Absence of functional P-glycoprotein in the blood–brain barrier leads to highly increased brain penetration of a number of important drugs. Depending on the pharmacological target of these drugs in the central nervous system (CNS), this can result in dramatically increased neurotoxicity, or fundamentally altered pharmacological effects of the drug. Given the variety of drugs affected by P-glycoprotein transport, it may be of tremendous therapeutic value to apply these insights to the development of drugs that should have either very poor or very good brain penetration, whichever is preferred for pharmacotherapeutic purposes. The clinical application of P-glycoprotein blockers should also be considered in order to improve the blood–brain barrier permeability of certain drugs that currently display insufficient brain penetration for effective therapy.

Keywords: Multidrug resistance genes; Pharmacokinetics; Neurotoxicity; Knockout mice; Brain capillary endothelial cells; P-Glycoprotein blockers; Drug transport

Contents

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0169-409X/99/$ – see front matter
PII: S0169-409X(98)00085-4

1. Introduction

Work of the last 9–10 years has unequivocally demonstrated that the drug-transporting (or mdr1-type) P-glycoproteins form an important part of the blood–brain barrier. Immunohistochemistry and analysis of isolated brain capillaries, primary cultures of brain capillary endothelial cells and immortalized cell lines derived from these cells have established that P-glycoprotein is present in the endothelial cells that form the blood–brain barrier, and functionally active in transporting drugs from the brain (or basolateral) side to the blood (apical or luminal) side of these cells. Subsequent analysis of knockout mice lacking P-glycoprotein in the blood–brain barrier and other animal models treated with P-glycoprotein blocking (and other) agents demonstrated that in vivo, blood–brain barrier P-glycoprotein can prevent the accumulation of many compounds, including a variety of drugs, in the brain. Many of the original findings in this field were recently reviewed by Naito and Tsuruo [1] and Tsuji and Tamai [2]. This review, therefore, aims to first discuss some general features of P-glycoprotein relevant to the understanding of its functioning in the blood–brain barrier, and the possible consequences of interfering with its activity in vivo. I will further focus on the recent advances that have been made in this area and on some remaining controversies, while referring to the earlier reviews for more detailed information.

2. General properties of P-glycoprotein

2.1. P-Glycoprotein and multidrug resistance

The drug-transporting P-glycoproteins were originally identified by their capacity to confer multidrug resistance to tumor cells against a range of anticancer drugs. The P-glycoprotein is localized in the plasma membrane of the cell, where it can actively extrude a variety of drugs from the cell, thus making it resistant to the cytotoxic activity of these drugs. The drug-transporting P-glycoproteins are *N*-glycosylated membrane proteins of about 1280 amino acids, the polypeptide chain consisting of two similar halves, each containing six putative transmembrane segments and an intracellular ATP-binding site (Fig. 1). Hydrolysis of ATP provides the energy for active drug export, which can occur against a large concentration gradient. For reviews see [3–7].

2.2. P-Glycoprotein substrates and blockers

The number and variety of drugs that can be transported by P-glycoprotein is truly staggering. They include not only anticancer drugs such as Vinca alkaloids, anthracyclines, epipodophyllotoxins, and taxanes, but also many other drugs, such as the

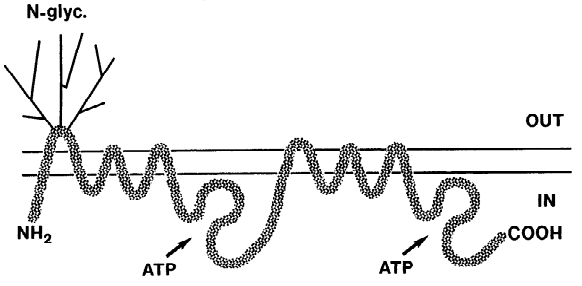

Fig. 1. Schematic two-dimensional representation of the putative transmembrane structural organization of human MDR1 P-glycoprotein (1280 amino acids long). The protein is primarily found in the plasma membrane of cells. The 12 transmembrane segments are thought to fold together and form a three-dimensional barrel-like structure in the membrane. Positions of the two intracellular ATP-binding sites are indicated with arrows. N- and C-terminus of the molecule are indicated. N-glyc. denotes the (extracellular) N-linked glycosylation trees of the protein.

immunosuppressive agent cyclosporin A, the cardiac glycoside digoxin, the glucocorticoid dexamethasone, the anthelmintic drug ivermectin, and several human immunodeficiency virus (HIV) protease inhibitors—to name but a few. It is as yet unclear how P-glycoprotein can recognize and transport such a structurally diverse set of compounds ranging in size from about 250 Da (cimetidine [8]) to more than 1850 Da (Gramicidin D). Many compounds contain aromatic groups, but non-aromatic linear or circular molecules are also transported. Most effectively transported compounds are basic or uncharged, but zwitterionic and negatively charged compounds (e.g., phosphatidylcholine analogues, methotrexate) can also be transported [9,10]. The only common structural denominator identified so far is that all transported P-glycoprotein substrates are at least somewhat hydrophobic and amphipathic in nature, i.e., containing spatially separated hydrophilic and hydrophobic moieties. These physical characteristics probably relate to the mechanism of drug translocation by P-glycoprotein, which may depend on the ability of the drug to insert into one hemileaflet of the membrane lipid bilayer. One favored (though as yet unproven) model proposes that P-glycoprotein transports its substrates mainly by 'flipping' them actively from the inner to the outer leaflet of the plasma membrane, which would result in a net efflux of drug [11]. Whatever the precise molecular mechanism of drug transport, P-glycoprotein activity can mediate very effective extrusion of drugs penetrating the plasma membrane, which results in very low intracellular drug levels.

In 1981, Tsuruo and co-workers [12] discovered that several compounds with low or even absent intrinsic cytotoxicity could effectively inhibit P-glycoprotein-mediated drug transport. Subsequent analysis revealed that many (though certainly not all) of these so-called reversal agents or P-glycoprotein blockers are in fact themselves transported substrates, which suggests that they inhibit in a competitive manner. They are as diverse in structure as the known P-glycoprotein substrates. Considering the potential clinical importance of P-glycoprotein-mediated drug transport in multidrug resistance of cancer cells, oral bioavailability, drug excretion, and brain penetration of drugs (see below), there is currently a flurry of activity to develop highly efficacious and specific P-glycoprotein blockers. PSC833, a non-immunosuppressive cyclosporin A analogue, is an effective and well-characterized representative of these blockers which is currently tested in Phase III trials for chemotherapy of Acute Myeloid Leukemia (see, e.g., [13,14]). Even more efficient and specific P-glycoprotein blockers may be in the pipeline [15–17].

2.3. P-Glycoprotein genes and tissue distribution

Apart from expression in the blood–brain barrier, drug-transporting P-glycoproteins occur in a range of other tissues. The most prominent sites are the apical membrane of intestinal epithelial cells of small and large intestine, the biliary canalicular membrane of hepatocytes, and the luminal membrane of proximal tubular epithelial cells in the kidney [18]. These locations suggest that mdr1-type P-glycoprotein may excrete its substrates into intestinal lumen, bile, and urine, respectively, thus eliminating these compounds from the body. High levels were further found in the adrenal gland of mice and humans (but not rats) and in the endometrium of pregnant uterus [18–20]. In addition, moderate levels of mdr1-type P-glycoprotein were found in a range of other tissues.

In contrast to man, which has only one drug-transporting P-glycoprotein gene, *MDR1*, mice and other analyzed rodents have two drug-transporting P-glycoprotein genes, *mdr1a* (also called *mdr3*) and *mdr1b* (also called *mdr1*) [21,22]. The substrate specificity of mdr1a and mdr1b P-glycoprotein is largely overlapping, although there are preferred drug substrates for each. The tissue distribution of mdr1a and mdr1b P-glycoprotein in the mouse is different but partly overlapping, and together the two mouse genes are expressed in roughly the same set of organs as the single human *MDR1* gene. This suggests that the mdr1a and mdr1b P-glycoproteins together perform the same set of functions in the mouse as MDR1 P-glycoprotein in man.

2.4. P-Glycoprotein knockout mice

In order to characterize the normal physiological function(s) of the mdr1-type P-glycoproteins, we have generated mice with a disruption of the *mdr1a*

gene, the *mdr1b* gene, or both of the *mdr1a* and *mdr1b* genes together [23,24]. These mice should also allow us to predict the consequences of complete and specific inhibition of the drug-transporting P-glycoproteins by administration of P-glycoprotein blockers. Fortunately, each of the three mouse strains was healthy and fertile, and did not display clear physiological abnormalities or a decreased life span. No abnormalities in anatomy, serum clinical chemistry, representation of lymphocyte classes, bile composition, and various other parameters could be found. Thus, under laboratory conditions, mdr1- type P-glycoproteins are not essential for the basic physiological functioning of the organism. In principle, this is good news for efforts to inhibit P-glycoprotein activity in humans, although we cannot exclude that humans may respond differently from mice to the absence of mdr1-type P-glycoprotein activity.

2.5. Pharmacological functions of the mdr1-type P-glycoproteins

As might be expected from the localization of the mdr1-type P-glycoproteins in intestine, liver, and kidney, and their capacity to transport many different drugs, mice lacking the mdr1-type P-glycoproteins display drastic alterations in the pharmacological handling of drugs. Analysis of these mice has demonstrated that the intestinal P-glycoprotein is an important factor in limiting the entry of substrate drugs from the intestinal lumen into the bloodstream, in other words, it can have a major negative effect on the oral availability of drugs [25,26]. Intestinal P-glycoprotein can further contribute to the direct excretion of drugs from the bloodstream into the intestinal lumen, whereas the bile canalicular P-glycoprotein contributes to the hepatobiliary excretion of drugs. Together these effects can result in a markedly slower elimination of drugs from the bloodstream, and in a clear shift from primarily fecal to primarily urinary excretion of some drugs [23,24,27–30]. These marked pharmacokinetic effects should be carefully considered when one tries to block P-glycoprotein activity in vivo with P-glycoprotein blockers for pharmacotherapeutic purposes.

3. Localization and activity of P-glycoprotein in the blood–brain barrier

3.1. Structure of the blood–brain barrier

The blood–brain barrier is physically formed by the blood capillary endothelial cells in the brain. In contrast to endothelial cells in capillary blood vessels in most other tissues, those in brain are closely joined to each other by tight junctions, and they cover the walls of the vessels as a continuous sheath, leaving no space between cells. Moreover, these endothelial cells demonstrate very little fenestration and pinocytosis (Fig. 2). As a result of this configuration, only very small hydrophilic molecules can enter the brain by diffusion past the tight junctions. All other molecules have to pass through the endothelial cells in order to enter the brain [31–33]. To meet the extensive metabolic needs of brain cells, the brain endothelial cells are, therefore, equipped with a variety of uptake and translocation systems for (amongst others) hydrophilic compounds like glucose and amino acids.

The characteristic differentiation state of brain capillary endothelial cells is at least in part induced and maintained by the close association of foot processes of brain glial or astrocyte cells with the basement membrane of the capillary [31,33,34]. These astrocytic foot processes or glial end foots ensheath most of the cylinder formed by the basement membrane of the capillary vessel (Fig. 2).

In principle, hydrophobic compounds like ethanol, caffeine, and nicotine can pass the blood–brain barrier by passive diffusion across the membranes of endothelial cells. In fact, for many hydrophobic compounds it was found that the degree of lipophilicity, corrected for the molecular weight, was a good predictor of the degree of brain penetration [35]. However, this rule only applied to molecules up to a molecular weight of about 400 Da. Several hydrophobic molecules between 400 and 700 Da entered the brain far less efficiently than expected, whereas even very hydrophobic molecules with a molecular weight above 700 Da did not enter the brain appreciably. It now appears that P-glycoprotein in the blood–brain barrier is a major factor explaining the low apparent brain penetration of hydro-

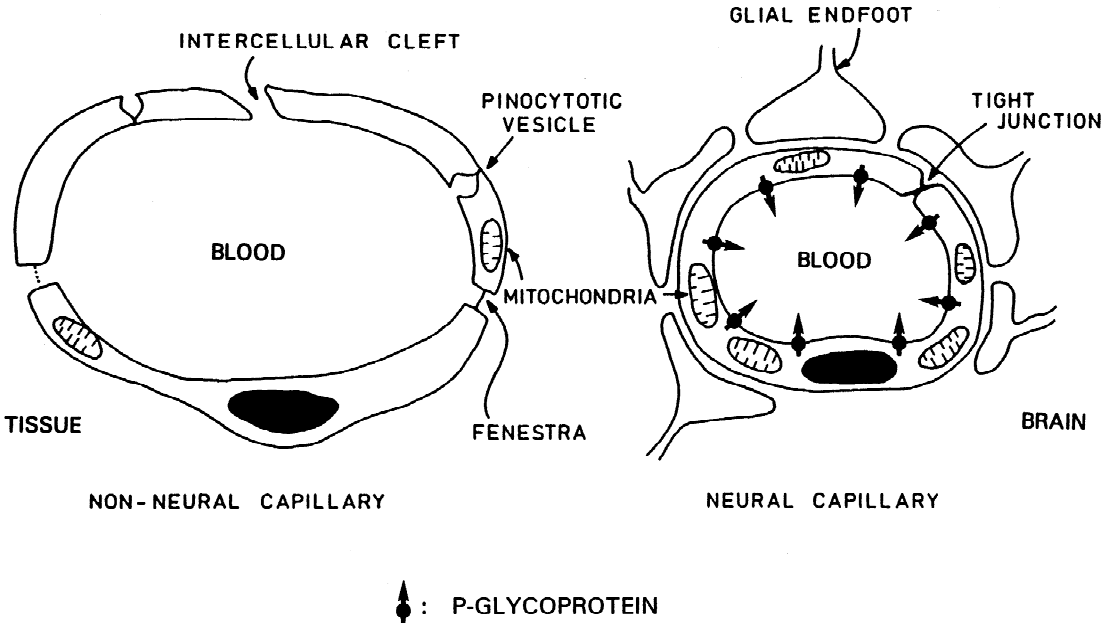

Fig. 2. P-glycoprotein localization and activity in the blood–brain barrier. The diagram shows a comparison of sections of typical blood capillaries in non-neural tissue and neural (brain) tissue. Unlike non-neural capillary endothelial cells, brain capillary endothelial cells are closely joined by tight junctions, and they display no intercellular clefts and little fenestration or pinocytosis. The balls-and-arrows indicate the localization of P-glycoprotein and the direction of drug transport in the luminal membrane of the brain capillary endothelial cells. For simplicity, the basal lamina, structural connective tissue surrounding the blood capillaries and separating the glial endfoots or astrocyte foot processes from the brain endothelial cells is not shown. Adapted from [42].

phobic compounds larger than 400/700 Da (see Section 4 below).

3.2. P-Glycoprotein in the blood–brain barrier

Interest in the possible function of P-glycoprotein in the blood–brain barrier was triggered by the findings of Cordon-Cardo et al. [36] and Thiebaut et al. [37] showing that several monoclonal antibodies recognizing P-glycoprotein specifically stained blood capillaries in brain (of human and rat), and to a lesser extent in testis, but not capillaries in most other tissues, or in the choroid plexus of the brain. These findings were subsequently corroborated by several other groups [38–41]. The resolution of light-microscopic immunohistochemistry is generally too low to allow precise subcellular localization of proteins in brain endothelial cells. However, based on the known drug transport properties of P-glycoprotein, it was suggested that P-glycoprotein in the blood–brain barrier would limit the entry of potentially toxic compounds from blood into the brain by pumping them actively back into the blood [36,37]. Considering the structure of the endothelial cells that make up the blood–brain barrier, it was reasonable to assume that P-glycoprotein would be localized in the blood luminal (apical) membrane (Fig. 2). When present in the luminal membrane, P-glycoprotein would be able to immediately extrude its substrates that had diffused into the endothelial cells back into the bloodstream. By sitting directly in the membrane that forms the lipophilic physical barrier, back-transport of compounds would be most efficient. A position in the luminal membrane would further be analogous to the position of P-glycoprotein in the apical membrane of intestinal epithelial cells, which also form a continuous barrier against entrance of compounds from the intestinal lumen into the bloodstream.

Since exact knowledge of P-glycoprotein localiza-

tion is essential for a proper understanding of the functioning of P-glycoprotein in the blood–brain barrier, I shall spend considerable space to discuss the experimental evidence currently available on this subject.

3.3. Localization of P-glycoprotein in brain capillary endothelial cells

Most of the structural work to date supports the idea that P-glycoprotein is primarily localized to the blood luminal membrane of brain capillary endothelial cells. By immunoelectron microscopy of brain sections, P-glycoprotein was detected exclusively at the luminal membrane using the monoclonal antibody MRK16 in human brain [39,43], and by the monoclonal antibody C219 in bovine brain [44]. Tanaka et al. [40] likewise detected a blood luminal localization of P-glycoprotein using monoclonal antibody C219 in endothelial cells of both normal human brain tissue and primary human glioma. In a different approach, Stewart et al. [45] used confocal immunofluorescence microscopy to demonstrate that C219 staining in endothelial cells of rat brain is localized to the luminal side of endothelial cell nuclei and not to the abluminal side.

It should be noted, though, that both light- and electron-microscopic immunohistochemistry is prone to many kinds of artefacts, which can result in both false-negative and false-positive results. In addition to the protein to which the antibodies are raised, antibodies may recognize other proteins that happen to share similar epitopes. For instance, the monoclonal antibody C219 was demonstrated to efficiently recognize at least one other protein that occurs in isolated mouse brain capillaries or in human and rat muscle and that is not a P-glycoprotein [37,46]. Illustrating another possible complication, the (extracellular) epitope of human MDR1 P-glycoprotein for the monoclonal antibody MRK16 can be completely shielded in some cell types by heavy *N*-glycosylation of P-glycoprotein [47]. Immunohistochemistry is further highly sensitive to optimal preparation and fixation of samples. Suboptimal fixation may lead to lack of recognition by obliteration of epitopes, or increased aspecific binding of the primary antibody [48]. Some preparations of anti-P-glycoprotein monoclonal antibodies have been shown to contain contaminating anti-A blood group antibodies [49]. These various complications have led immunohistochemistry experts to state that immunolocalizations can only be considered reliable when consistent results are obtained with at least two, and preferably three or more different antibodies [38].

An excellent and thorough study by Beaulieu et al. [50], employing a novel technique, provided the most convincing evidence so far that in capillary endothelial cells of rat brain, P-glycoprotein is predominantly, if not exclusively, localized to the luminal membrane. After selective binding of colloidal silica particles to the luminal membrane of endothelial cells (by injection of these particles into an intact vascular bed), and coating of the particles with a polyanion, luminal membranes could be specifically purified by density centrifugation of homogenized brain tissue. This procedure resulted in a luminal membrane preparation with a 10-fold enrichment of the brain endothelial membrane marker protein GLUT1 relative to isolated brain capillaries, and a 17-fold enrichment of P-glycoprotein. Enrichment of these proteins relative to whole brain membrane preparations was 240- and 400-fold, respectively. The protein GFAP, a specific marker for astrocytes, was enriched only 1.4- and 2.6-fold relative to brain capillaries and whole brain membranes, respectively, indicating that astrocytes formed at best a minor contamination of the luminal membrane preparation. The concentration of integrinαv, a marker for anti-luminal endothelial membranes, was decreased to 0.4- and 0.3-fold the levels in isolated brain capillaries and whole brain membranes, respectively, demonstrating that very little anti-luminal membrane contaminated the luminal membrane preparation. The efficient enrichment of P-glycoprotein in the luminal membrane preparation can only be explained by assuming that a high level of P-glycoprotein is normally present in the luminal membrane of brain endothelial cells.

These authors took great care to validate that the protein they detected on Western blots was indeed P-glycoprotein: they used two independent antibodies, the monoclonal antibody C219 and the polyclonal antibody Ab-1, which gave identical results. The identity of the recognized protein was further verified by characteristic mobility shifts upon en-

zymatic deglycosylation, and photo-affinity labeling with the P-glycoprotein substrate iodoarylazidoprozasin. Altogether, this study leaves very little doubt that in rat brain, the luminal membrane of the brain capillary endothelial cells is a primary site of P-glycoprotein localization.

A few studies provided data suggesting that P-glycoprotein in humans and some primates can be present in brain cells other than endothelial cells, and may even be absent from the luminal membrane of the endothelial cells. Tishler et al. [51] found strong immunostaining with the anti-P-glycoprotein monoclonal antibody JSB-1 of blood capillaries in human brain but, in addition, in brain samples of many patients with intractable epilepsy, P-glycoprotein was also detected in astrocytes. However, in normal brain samples (three out of three) astrocyte staining was not observed.

Pardridge et al. [52] observed that in isolated human brain capillaries the anti-P-glycoprotein antibodies MRK16 and C219 bound to microvessels with a similar, discontinuous staining pattern as a polyclonal antiserum directed against the astrocyte-specific marker protein GFAP. In addition, in brain sections of rhesus and squirrel monkey, MRK16 stained both brain capillaries and astrocyte processes. The capillary staining appeared again discontinuous. Immunostaining with the anti-GFAP antiserum also yielded staining of blood capillaries in some fields, and staining of astrocyte foot processes in all fields, resulting in a qualitatively comparably staining pattern between the two antibodies. These results suggested that MRK16 could primarily recognize an antigen in astrocyte foot processes. Since these foot processes are tightly associated with the basement membrane of brain capillaries, (and remain so even after isolation of the capillaries; see Fig. 2), this would result in an apparent staining of endothelial cells at the resolution of light-microscopic immunohistochemistry.

Subsequent double immunolabeling and confocal light microscopy performed on isolated human brain capillaries with MRK16 and anti-GFAP antiserum supported this model [52]. MRK16 staining was found to be discontinuous and primarily on the abluminal side of the endothelial cells, colocalizing completely with GFAP immunostaining. In contrast, staining for the endothelial membrane marker protein GLUT1 was continuous and showed only minimal overlap with MRK16 staining. Finally, intravenous administration of ^{125}I-labeled MRK16 to a squirrel monkey did not result in appreciable sequestration of the labeled compound in the brain vascular bed, indicating that there was no extensive binding of the antibody to the capillary luminal membranes.

In summary, the similarity in immunostaining by MRK16 and anti-GFAP antiserum, the difference with immunostaining by anti-GLUT1 antiserum, the apparently discontinuous and abluminal localization of MRK16 staining in isolated brain capillaries, and the absence of MRK16 binding to the brain endothelial vascular bed lead the authors to propose that P-glycoprotein is not present in the luminal membrane of brain capillary endothelial cells, but rather primarily localized to astrocyte foot processes.

It is clear that it is difficult to reconcile the data from this latter study [52] with the results of most of the other immunohistochemical studies. Also in view of functional analysis of P-glycoprotein localization and activity in cultured brain capillary endothelial cells and in mice lacking blood–brain barrier P-glycoprotein (see below), it does not seem probable that P-glycoprotein is absent from the blood luminal membrane. However, it may be that the localization of blood–brain barrier P-glycoprotein in squirrel and rhesus monkey and possibly humans is different from that in rodents, although I consider this unlikely. It would imply a fundamentally different functional role of blood–brain barrier P-glycoprotein in rodents and primates. It is further clear that the main conclusions of Pardridge et al. [52] regarding P-glycoprotein localization are essentially based on the binding behavior of only one monoclonal antibody, MRK16. The possible limitations of data obtained with just one antibody have been outlined above. This study did not provide independent proof that the antigen as detected here by MRK16 was indeed (only) P-glycoprotein. Moreover, it is possible that extensive glycosylation of P-glycoprotein in the blood luminal membrane may have prevented efficient binding of MRK16.

At this point, therefore, in my opinion, the balance of experimental evidence for blood–brain barrier localization of P-glycoprotein in humans is that it is most likely present in the blood luminal membrane of the brain capillary endothelial cells, although this

does not preclude that it can also occur in astrocytes, especially in certain pathological states [51]. Nevertheless, substantial additional work will have to be done, using various experimental approaches such as immunoelectron microscopy with at least two independent monoclonal antibodies, and possibly in situ RNA hybridization, to establish conclusively whether P-glycoprotein is present or absent in astrocyte foot processes of normal human brain and, more importantly, in the blood luminal membrane of human brain capillary endothelial cells. It would further be useful to consider applying the technique developed by Beaulieu et al. [50] to rhesus or squirrel monkeys or other primates.

3.4. P-Glycoprotein activity in cultured brain capillary endothelial cells

The first experimental evidence that blood–brain barrier P-glycoprotein was potentially involved in drug transport came from studies with cultured brain endothelial cells [44,46,53–58]. P-Glycoprotein was detected in immortalized mouse brain capillary endothelial cell lines, or primary cultures of bovine, porcine, or murine brain capillary endothelial cells, and reduced cellular accumulation of typical P-glycoprotein substrate drugs was demonstrated, which could be reversed by P-glycoprotein blocking agents. When grown as polarized cell layers, these cells displayed increased basolateral (abluminal) to apical (luminal) transport of vincristine and cyclosporin A [53,59], suggesting apical localization and transport activity of P-glycoprotein. Indeed, immunostaining with various monoclonal and polyclonal antibodies demonstrated an exclusively apical localization of P-glycoprotein in cultured polarized mouse [53], bovine [44], and human [58] brain endothelial cells.

It should be remarked, though, that cultured brain capillary endothelial cells do not necessarily reflect exactly all the differentiation properties of in situ brain capillary endothelial cells. For instance, extensively cultured or immortalized mouse or rat brain capillary endothelial cells contain primarily or only mdr1b P-glycoprotein, whereas in vivo mouse and rat brain capillaries appear to contain only mdr1a P-glycoprotein [23,53,57]. Barrand et al. [57] demonstrated that upon prolonged culturing, rat brain capillary endothelial cells lose *mdr1a* expression and gain *mdr1b* expression. Results obtained with these systems should, therefore, be extrapolated with caution to the situation in the blood–brain barrier in vivo.

4. The in vivo impact of blood–brain barrier P-glycoprotein

4.1. Ivermectin hypersensitivity of mice lacking mdr1a P-glycoprotein in the blood–brain barrier

The real impact of P-glycoprotein in the blood–brain barrier became only evident with the generation of knockout mice lacking mdr1a P-glycoprotein (*mdr1a* (−/−) mice). As a result of this knockout, these mice lack detectable P-glycoprotein in the brain capillary endothelial cells [23]. The consequences are dramatic. Whereas the mice behave perfectly normal under average laboratory conditions, they turned out to be almost 100-fold more sensitive to the neurotoxic pesticide ivermectin. This was discovered by chance, as the mice were sprayed with ivermectin to treat a mite infestation, normally a very safe routine procedure. In this case, however, nearly all *mdr1a* (−/−) mice died. Subsequent analysis demonstrated that the *mdr1a* (−/−) mice accumulated nearly 100-fold more [^{3}H]ivermectin in their brains than wild-type mice, whereas the plasma levels were only increased by about 3-fold. Ivermectin was next shown to be a good transported substrate for mouse mdr1a and human MDR1 P-glycoprotein [60]. The results indicated that blood–brain barrier P-glycoprotein can have a major impact in excluding substrate drugs from the brain compartment, and that this can have dramatic consequences for the pharmacological activity of a drug.

Ivermectin is not just a veterinary pesticide. Due to its efficiency as an anthelmintic agent, it is currently the drug of choice to treat river blindness (onchocerciasis), a debilitating tropical disease caused by a parasitic worm. More than 10 million people have been treated with this drug, apparently without clear adverse side effects. This suggests that people with a genetic deficiency in blood–brain barrier P-glycoprotein, if they exist at all, are rare. That spontaneous occurrence of genetic mutations of

blood–brain barrier P-glycoprotein is not a merely theoretical possibility is illustrated by the recent identification of an inbred subpopulation of CF1 mice that have a mutation in their *mdr1a* gene [61,62]. These mice behave virtually identically to the *mdr1a* knockout mice with respect to ivermectin tissue distribution, and they are 100-fold hypersensitive to the ivermectin analogue, abamectin. Moreover, a subpopulation of Collie dogs also displays about 100-fold hypersensitivity to ivermectin, concomitant with a highly increased brain penetration of ivermectin [63]. It is very likely that these dogs have a genetic deficiency in their blood–brain barrier P-glycoprotein similar to that in *mdr1a* knockout mice.

4.2. Effects of other drugs in mdr1a knockout mice

These studies were then extended to a range of other drugs known to be P-glycoprotein substrates. The anticancer drug vinblastine accumulated 20-fold more in brain of *mdr1a* knockout mice, whereas the plasma level was only 2-fold increased [23]. Similar results were obtained with the ^{3}H-labeled drugs digoxin and cyclosporin A, while more moderate increases in brain concentration were found for [^{3}H]morphine and [^{3}H]dexamethasone [60]. The drug digoxin, which has a strong tendency to bind to brain tissue, continued to accumulate in the brain of *mdr1a* knockout mice for a period of 3 days after a single intravenous bolus injection, resulting in a 200-fold higher brain level as compared to wild-type mice (see Fig. 3 and Ref. [28]). Unlike ivermectin, drugs like vinblastine, cyclosporin A and digoxin have little toxic activity in the brain of mice, which prevented unacceptable increases in CNS toxicity in the *mdr1a* (−/−) mice. However, the murine form of the Na/K-ATPase, the target enzyme of digoxin, is unusually resistant to the pharmacological and toxic action of the drug. In contrast, the human form is highly sensitive, so we would expect major CNS toxicity of digoxin in humans without functional P-glycoprotein in their blood–brain barrier.

That blood–brain barrier P-glycoprotein can have a decisive effect on the clinical application of drugs other than ivermectin was suggested by the results obtained with domperidone and loperamide. Both drugs are efficiently transported by mdr1a and MDR1 P-glycoprotein [64]. Domperidone is a dopamine antagonist which has unexpectedly low activity in the brain as it does not pass the blood–brain barrier. As a consequence, it cannot be used as a neuroleptic (anti-psychotic) drug like other dopamine antagonists, but it can be used as an anti-emetic drug due to its selective peripheral activity. When domperidone was administered to *mdr1a* (−/−) mice, they displayed extreme passivity and total lack of spontaneous movement, indicative of a CNS activity of domperidone, which was not observed in wild-

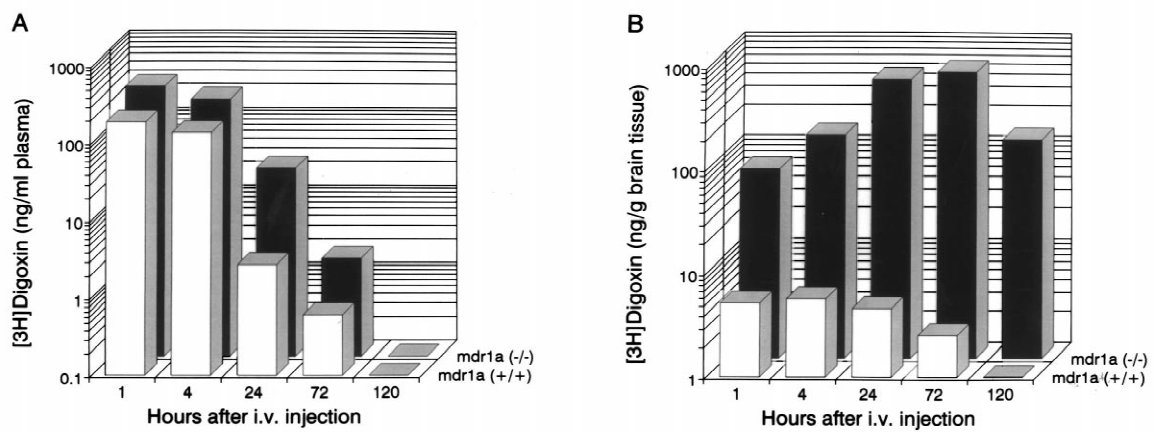

Fig. 3. Levels of [^{3}H]digoxin-derived radioactivity in plasma (A) and in brain (B) of wild-type mice (*mdr1a* (+/+), white bars) and *mdr1a* (−/−) mice (black bars) at various time points after a single intravenous administration of [^{3}H]digoxin (0.2 mg/kg). Data are expressed as ng [^{3}H]digoxin equivalent per ml plasma or per g tissue. Adapted from Ref. [28].

type mice given similar doses [64]. This result suggests that blood–brain barrier P-glycoprotein is a major determining factor for the clinical use of domperidone.

The drug loperamide (Imodium®) is structurally an opiate like morphine and related compounds. However, in humans and in animal models, loperamide displays only peripheral opiate-like effects on the gastrointestinal tract, leading to constipation, whereas pharmacological effects in the CNS are hardly ever observed. As a consequence, loperamide is used as a highly effective, over-the-counter antidiarrheal drug. When loperamide was administered to *mdr1a* (−/−) mice, they displayed a full-blown picture typical of opiate effects in the CNS in mice: pronounced excitement, compulsive circling movements interrupted by bouts of immobility, a crouched appearance and a characteristically erected tail on an arched back ('straub tail'). Wild-type mice merely demonstrated passivity. Upon administration of [^{3}H]loperamide, the *mdr1a* (−/−) mice accumulated 13-fold higher levels of radioactivity in the brain, whereas the plasma level was only 2-fold higher than that in wild-type mice [64]. These results clearly suggest that without P-glycoprotein in the blood–brain barrier, loperamide would be a centrally-active opiate which could probably not be obtained over-the-counter.

Drugs like ivermectin, domperidone, and loperamide have their pharmacological targets in brain neuronal cells. The fact that absence of blood–brain barrier P-glycoprotein leads to a dramatically increased sensitivity to these drugs proves that P-glycoprotein is directly involved in the protection of the neuronal compartment of the brain. The simplest explanation for the findings is that P-glycoprotein reduces the brain interstitial fluid concentration of these drugs by blocking their entry at the level of the capillary endothelial cells.

A potential complication in the analysis of knockout mice is that, as a consequence of the constructed genetic alteration, there may be changes in the expression of other genes, or there may be unforeseen defects that go beyond the mere absence of the protein encoded by the disrupted gene. We found for instance that *mdr1a* (−/−) mice displayed a 5-fold upregulation of *mdr1b* expression in liver and kidney, but not in other tissues analyzed [23]. There could, however, be additional changes, such as the overexpression or loss of expression of other transporter genes in the blood–brain barrier. One serious concern is that the physical integrity of the blood–brain barrier might be compromised in *mdr1a* knockout mice, leading to a damaged, 'leaky' endothelial cell layer. However, for a range of both hydrophilic and hydrophobic drugs such as [^{3}H]methotrexate, [^{14}C]topotecan, [^{3}H]oxytocin, [^{14}C]phenytoin, [^{3}H]haloperidol, and [^{3}H]clozapine, we did not observe any change in brain penetration in the *mdr1a* or *mdr1a/1b* (−/−) mice ([64] and our unpublished results), indicating that it is highly unlikely that the physical structure of the blood–brain barrier is substantially altered in these knockout mice.

By now, many more drugs have been shown to accumulate to higher extents in the brains of *mdr1a* (−/−) or *mdr1a/1b* (−/−) mice. Table 1 lists some representative examples. The recent finding that the brain penetration of the human immunodeficiency virus (HIV-1) protease inhibitors indinavir, nelfinavir and saquinavir is also very markedly affected by mdr1a P-glycoprotein is of particular interest [65,66]. These drugs have resulted in a drastic improvement in the therapy of AIDS. However, the CNS potentially represents a sanctuary for HIV-1 infection, and poor penetration of the protease inhibitor drugs in the brain might turn out to

Table 1
Drugs affected by P-glycoprotein in the blood–brain barrier

Drug	M_r	Therapeutic category
Ondansetron	293	Antiemetic
Dexamethasone	392	Glucocorticoid
Domperidone	426	Antiemetic
Loperamide	477	Antidiarrheal
Doxorubicin	544	Antineoplastic
Nelfinavir	568	HIV protease inhibitor
Indinavir	614	HIV protease inhibitor
Saquinavir	671	HIV protease inhibitor
Erythromycin	734	Antibiotic
Digoxin	781	Cardiotonic
Vinblastine	811	Antineoplastic
Paclitaxel	854	Antineoplastic
Ivermectin	874	Anthelmintic, pesticide
Cyclosporin A	1203	Immune suppressant

Molecular sizes and therapeutic categories of some drugs with markedly increased brain penetration and/or CNS toxicity in P-glycoprotein knockout mice [23,60,64,65,78,79].

be of importance in limiting the effective treatment of AIDS.

It is clear from the above-mentioned results and from the huge diversity of compounds that can be transported by P-glycoprotein, that there will be far more drugs or candidate drugs for which the brain penetration will also be affected by blood–brain barrier P-glycoprotein. For many of these drugs, this may have important negative or positive consequences for their pharmacotherapeutic applications. In view of its pharmacological impact, it may, therefore, be practically extremely useful to block blood–brain barrier P-glycoprotein transiently, in order to improve entry of therapeutic compounds into the brain when so desired. The following section discusses some of the progress that has been made in testing the feasibility of this approach.

5. Controlled modulation of P-glycoprotein activity in the blood–brain barrier

5.1. Experiments in normal mice and rats

As soon as it was recognized that P-glycoprotein in the blood–brain barrier might be an important determinant of the brain penetration of many drugs, attempts were initiated to enhance the brain penetration of drugs by administration of P-glycoprotein blockers. Some initial negative results in these attempts (see e.g., [67]) could be explained by the use of relatively inefficient P-glycoprotein blockers, the use of suboptimal administration protocols of the blocker, or a combination of these factors. It should also be noted that, based on theoretical considerations, it may be easier to block P-glycoprotein-mediated transport of some drugs than others when using the same blocker. If the affinity of a given drug for P-glycoprotein is markedly higher than the affinity of the blocker, it may be difficult to obtain sufficient plasma concentrations of the blocker in plasma for effective inhibition of blood–brain barrier P-glycoprotein. Nonetheless, several groups could demonstrate substantial blocking effects.

Sakata et al. [68] showed that quinidine infused through a microdialysis probe into rat brain could enhance the brain extravascular extraction of cyclosporin A about 2.5-fold in an in situ brain perfusion model. Wang et al. [69] found that the brain extracellular fluid levels of the P-glycoprotein substrate rhodamine 123 as measured by microdialysis could be increased 3- to 4-fold by intravenous infusion of cyclosporin A. Chikhale et al. [70] found substantial increases in the brain uptake of hydrophobic model peptides by the coinjection of very high concentrations (0.05–0.5 mM) of verapamil in a rat brain in situ perfusion model.

The availability of the efficient P-glycoprotein-blocker PSC833 resulted in a clear improvement in the possibility to block blood–brain barrier P-glycoprotein with clinically realistic plasma concentrations of the blocker. For instance, intravenous bolus administration of PSC833 could enhance the brain/blood concentration ratio of cyclosporin A 5-fold [71]. In an in situ brain perfusion model, the brain uptake of colchicine and vinblastine was increased 8- and 9-fold, respectively, after an intravenous bolus injection of PSC833 [72] and in a continuous intravenous infusion model, PSC833 enhanced the brain levels of colchicine at least 10-fold as measured by brain microdialysis, whereas the plasma level was increased only about 2-fold [73]. Interestingly, in the experiments where subcompartments of the brain were tested, the choroid plexus did not display differences, in contrast to the other ('gray') areas of the brain [72,74]. This is in line with the absence of detectable P-glycoprotein staining in the choroid plexus [36]. Didier and Loor [75] demonstrated that co-administration of ivermectin and PSC833 to mice results in an at least 10-fold increased neurotoxicity of ivermectin, although these authors did not directly show that this was primarily due to increased brain penetration of ivermectin, or possibly also to delayed elimination of the drug from plasma.

5.2. Experiments in P-glycoprotein knockout mice

A principal limitation of the above-mentioned experiments is that, in the absence of a P-glycoprotein-negative control, it is uncertain to what extent blood–brain barrier P-glycoprotein activity was inhibited. We, therefore, used the *mdr1a/1b* knockout mice in order to study to what extent orally administered PSC833 could enhance the brain penetration of digoxin [29]. Part of the reason for choosing oral

administration of PSC833 (in contrast to the studies discussed above) was that for possible future clinical applications, it would be preferable to administer P-glycoprotein blockers by the oral route. We found that the absolute accumulation of digoxin in the brain was increased 19-fold in PSC833-treated wild-type mice, but since the plasma concentration was also raised about 2.4-fold (due to the inhibition of hepatobiliary and intestinal excretion of digoxin), this resulted in an 8-fold increase in brain/plasma ratio (see Fig. 4). In *mdr1a/1b* (−/−) mice treated with PSC833, the brain-to-plasma ratio of digoxin was only 1.6-fold higher than in PSC833-treated wild-type mice at a comparable plasma concentration, indicating that we could obtain nearly complete inhibition of blood–brain barrier P-glycoprotein with oral PSC833. It should be noted, though, that PSC833 diminished the distribution of digoxin to the brain of *mdr1a/1b* (−/−) mice relative to untreated *mdr1a/1b* (−/−) mice 2-fold, by an as yet unknown mechanism. Thus, the brain concentration of digoxin in PSC833-treated wild-type mice was still 3.6-fold lower than that in untreated knockout mice lacking P-glycoprotein.

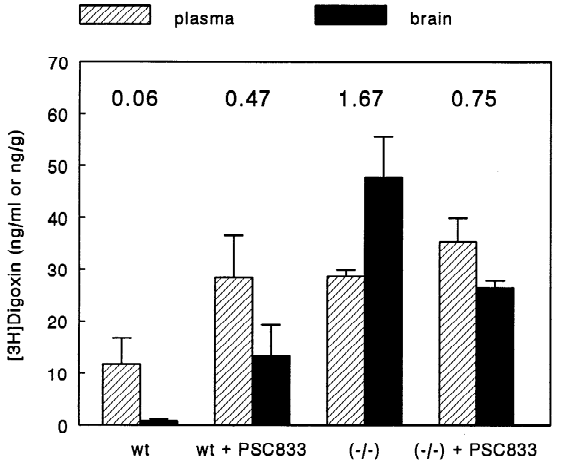

Fig. 4. Increased brain penetration of [^{3}H]digoxin by oral treatment with PSC833. Wild-type or *mdr1a/1b* (−/−) mice received oral PSC833 (50 mg/kg) or only vehicle, and 2 h later [^{3}H]digoxin (0.05 mg/kg) was administered intravenously. Plasma (hatched bars) and brain levels (black bars) of [^{3}H]digoxin-derived radioactivity were determined 4 h after digoxin administration. The average brain/plasma ratio for each treatment group is indicated over each couple of bars. wt denotes wild-type mice, and (−/−) *mdr1a/1b* (−/−) mice. Data are adapted from Ref. [29].

Although these interactions are probably typical for certain combinations of drugs and blocking agents, they illustrate that it may be difficult to always obtain complete and 'uncomplicated' inhibition of blood–brain barrier P-glycoprotein in a clinically realistic situation. Nevertheless, it is clear that a very substantial improvement of the brain penetration of drugs can be achieved by using effective P-glycoprotein blockers, and it is very likely that this improvement can be of great therapeutic value for some drugs.

6. Conclusions and caveats

In principle, almost any of the experimental approaches used so far in the analysis of blood–brain barrier P-glycoprotein may have its complications. Immunohistochemistry can be prone to false-positive and false-negative results, and cultured brain capillary endothelial cells may lose or alter part of their characteristic differentiation properties. P-Glycoprotein knockout mice may have undergone additional changes secondary to the loss of P-glycoprotein expression, and inhibitors of P-glycoprotein may not be completely specific. Nevertheless, the general consistency of almost all of the data obtained so far leaves very little doubt that P-glycoprotein is an important gatekeeper in the blood–brain barrier that prohibits the entry of a range of hydrophobic amphipathic drugs into the brain.

Of course, there is much more to the blood–brain barrier than just a continuous lipophilic barrier and P-glycoprotein. For instance, there may well be additional transporters in the brain capillary endothelial cells that can also contribute to the extrusion of hydrophobic drugs (see e.g., [2]), and this review has not addressed the multitude of specific uptake and transport systems that exist in the blood–brain barrier. Nevertheless, this review clearly demonstrates that for many drugs, P-glycoprotein is a major determinant for brain penetration.

There are several obvious practical consequences of the knowledge now gained about the blood–brain barrier P-glycoprotein function:

(1) Probably of most immediate concern is that several clinical trials are currently running to improve the response of (non-CNS) cancers expressing

MDR1 P-glycoprotein to chemotherapy, by co-administration of effective P-glycoprotein blockers (see e.g., [13]). These trials should be carefully monitored for inadvertent side effects in the CNS of the primary chemotherapeutic drugs, or of other drugs administered at the same time to the patients, since the plasma levels of the blocker used may be high enough to affect blood–brain barrier P-glycoprotein.

(2) In many cases, targets for pharmacotherapy are positioned behind the blood–brain barrier. This is for instance the case with most dysfunctions of brain neuronal cells, viral infections that penetrate the brain (e.g., HIV), and possibly some brain tumors, especially gliomas (see e.g., [40,41]. Optimal treatment of these afflictions is severely limited by the difficulty to obtain sufficient concentrations of the therapeutic drug at the target site. When potentially effective drugs are known to be good P-glycoprotein substrates, it may be useful to try and improve the brain penetration of these drug by co-administration of effective P-glycoprotein blockers. Experiments in mice suggest that it may be feasible and acceptable to obtain a substantial increase in brain penetration by this procedure. Obviously, great care must be exercised in such protocols in order to prevent inadvertently increased CNS toxic side effects of the drug itself, or of other drugs simultaneously administered to the patient.

(3) An alternative option is to try and design drugs such that they are either good P-glycoprotein substrates if it is desirable that they do not enter the brain, or poor P-glycoprotein substrates if it is desirable that they do enter the brain. Examples of the first group could be antihistaminics for treatment of allergies, which should be minimally sedative, examples of the second group chemotherapeutic agents for treatment of brain tumors. A complication for such an approach is that it is still not clear what exactly defines whether a compound is a well transported substrate for P-glycoprotein [76,77]. Clearly, for a number of purposes it would be highly desirable to make an accurate prediction just based on the chemical structure of the drug. Pharmaceutical companies would be wise to invest considerable efforts in solving this problem once and for all. This is all the more important since the oral bioavailability of drugs can also be drastically affected by P-glycoprotein activity [25,26].

In summary, in my opinion, the insights now gained in the function of blood–brain barrier P-glycoprotein hold many promises for further optimization of pharmacotherapy of both diseases localized in the brain and elsewhere in the body. Future research will reveal to what extent these promises can be fulfilled, and I expect that the availability of P-glycoprotein knockout mice will be of great help in finding the best possible applications of our recently gained knowledge.

Acknowledgements

I thank Mr J.W. Jonker and Dr J. Allen for critical reading of the manuscript. Most of the work at The Netherlands Cancer Institute described in this review was supported by grants NKI 92-41 and NKI 97-1434 of the Dutch Cancer Society.

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