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Blood-Brain Barriers

From Ontogeny to Artificial Interfaces

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The Blood-Brain Barrier: Roles of the Multidrug Resistance Transporter P-Glycoprotein

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19.1
Introduction

For the brain, the blood-brain barrier (BBB) formed by the brain capillary endothelial cells (BCEC) is considered to be the major route for the uptake of endogenous and exogenous ligands into the brain parenchyma [1]. The EC of brain capillaries are closely sealed by tight junctions and constitute a continuous endothelium. Moreover, brain capillaries possess few fenestrae or endocytic vesicles as compared to the capillaries of other organs [1–3]. BCEC are surrounded by astrocytes, pericytes, microglial cells and by the extracellular matrix. The close association of BCEC with the astrocyte foot processes and the basement membrane of capillaries is important for the development and maintenance of the BBB properties that permit tight control of the blood-brain exchange of molecules [1–4].

The restrictive nature of the BBB is due, in part, to the tight junctions that prevent significant passive movement of small hydrophilic molecules between blood and brain. Nutrients such as glucose and amino acids penetrate into the brain via transporters, whereas uptake of larger molecules, including insulin and transferrin, occurs via receptor-mediated endocytosis [5, 6]. Among the factors controlling the passive entry of drugs into the CNS, lipid solubility is the predominant element because of the lipidic nature of cell membranes [7]. The overall hydrophilic/lipophilic balance of a molecule appears to be a better predictor of BBB permeability than the octanol/buffer partition coefficient. Molecular size, to which the rate of solute diffusion is inversely related, also appears to be relevant for hydrophilic compounds, but does not significantly influence the BBB permeability of lipophilic compounds. Aside from passive diffusion through lipid membranes, the binding of molecules to plasma proteins, ionization at physiological pH (pK_a), affinity and capacity of transport systems and potential BBB/cerebral metabolism are also important for entry into the brain. There are also an increasing number of studies showing that the activity of the

efflux transporter P-glycoprotein (P-gp) at the BBB prevents significant accumulation of many hydrophobic molecules or drugs in the CNS [8, 9].

19.2 The Multidrug Transporter P-Glycoprotein

Almost two decades ago, Juliano and Ling showed that "Drug-resistant Chinese hamster ovary cell membranes possess a carbohydrate-containing component of 170 000 daltons apparent molecular weight which is not observed in wild type" [10]. They were the first to demonstrate that overexpression of a glycoprotein they called P-glycoprotein (P for permeability) was responsible for pleotropic resistance of tumor cells against a wide variety of chemotherapeutic agents. Thus, the development of simultaneous resistance to multiple drugs (MDR) that occurs after selection for resistance to a single agent mimics the MDR of human tumors treated with chemotherapy. Therefore the ATP-dependent efflux pump of anticancer drugs, P-gp, is one of the main causes of failure in chemotherapy.

P-gp is a member of the ATP-binding cassette (ABC) group of transporters which represent the largest family of transmembrane proteins. They are found in all prokaryotic and eukaryotic cells. The vast majority of ABC proteins are active transporters requiring ATP hydrolysis to provide a driving force to translocate substrates against a concentration gradient across cell membranes. The first ABC transporter was sequenced more than 20 years ago [11]. Proteins are classified as ABC transporters based on the sequence and organization of their ATP-binding domains, also known as nucleotide-binding folds (NBFs). The NBFs contain characteristic motifs (Walker A and B motifs), which are found in all ATP-binding proteins [12]. Some 48 members of the ABC superfamily have been described in humans [13, 14]. They are divided into seven families (ABC A–G). The standard nomenclature, developed by the Human Genome Organization, is available at http://www.gene.ucl.ac.uk/nomenclature/genefamily/abc. Three of the ABC subfamilies (C, B, G) contain transporters known to express significant transport activity in the BBB and in the blood-cerebrospinal fluid barriers (BCSFB): P-gp (ABCB1), MRP (ABCC1, 4, 5, 6) and BCRP (ABCG2). These play a pivotal role in the brain barrier by functioning as active efflux pumps.

19.2.1 P-gp Isoforms

Among the three efflux protein families known to have activity at the BBB, P-gp is one of the important ones identified at the BBB. It has been proposed that P-gp plays an important role at the BBB in limiting the entry of many drugs into the brain [15, 16]. The expression of P-gp in human BBB endothelial cells was first described by Cordon-Cardo et al. and Thiebaut et al. in 1989 [17, 18]. P-gp is encoded by the *MDR1* gene in humans and by *mdr1a* and *mdr1b* in rodents and is associated with the MDR phenotype. P-gp encoded by *MDR2* in humans or by *mdr2* in rodents does not play an important role in the transport of drugs [19]. Mice genetically deficient in the *mdr1b* gene or in both *mdr1a* and *mdr1b* genes have normal viability. However, they show an accumulation of various drugs in the brain and other tissues, indicating that P-gp may act as a guardian by preventing the passage and accumulation of many drugs in the brain [9, 20, 21]. Moreover, it was shown that P-gp could limit the access of naturally occurring molecules, such as the glucocorticoid cortisol, to the mouse and human brain, particularly to the hippocampal area [22, 23].

19.2.2 Structure

The P-gp human product of *MDR1* has 1280 residues whereas the mouse proteins *mdr* or *mdr3* have 1276 residues. A model for the structure of P-gp has been proposed based on the amino acid sequences (Fig. 19.1). In this model, two homologous halves (43% homology between the cDNA sequences) comprise each membrane transporter. Each half contains six alpha-helix transmembrane-spanning segments (TMs), for a total of 12 TMs. One cytoplasmic domain containing an ATP-binding site is also found on each half of the molecule. Phosphorylation sites have been identified in the linker region between the two halves of MDR1 in human [24] and two to four glycosylation sites have been described in the first extracellular loop between TM1 and TM2. Thus, the variability in the measured molecular weight of P-gp (150–180 kDa) is presumably due to different levels of P-gp glycosylation between species and tissues [24–26].

Using different biochemical approaches, various oligomeric states have been reported for P-gp, depending on the tissue and cells employed [27, 28]. Electron microscopy and computer models proposed that association between the TMs of P-gp leads to the formation of a pore with a funnel-shape across the membrane [29]. In this 3-D model, the protein viewed from the extracellular side has a diameter of about 10 nm surrounding a central pore of 5 nm diameter. The opening of this pore is narrowed inside the membrane by the nucleotide-binding domain of the protein. A rearrangement of the human P-gp TMs in the presence of different substrates or as a result of ATP hydrolysis has also been proposed [30–33]. From their studies, Loo and Clarke proposed a number of amino acid residues that could be involved in the interactions of, and thus the binding domains for, various P-gp substrates. Data obtained with transport measurements of fluorescent substrates and photoaffinity labeling studies support the existence of different binding sites for P-gp substrates, inhibitors or modulators [34–36]. Recently, a crystallographic structure of P-gp has been reported which demonstrates that, upon binding ATP, the TMs undergo a reorganization in compact domains [37]. In addition, models representing two different functional states of P-gp (nucleotide-free, nucleotide-bound) have been proposed for the conformational rearrangement of TMs [38]. Despite the voluminous data on

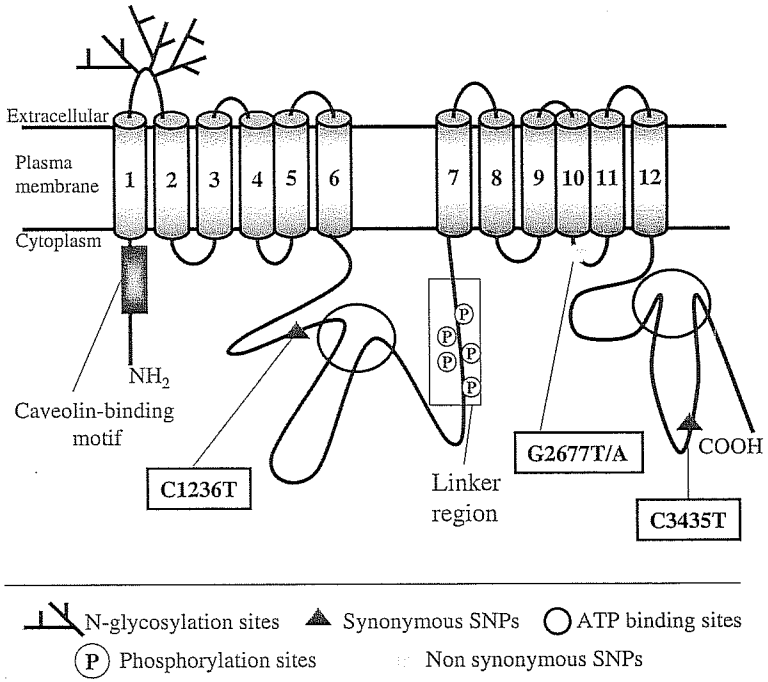

Fig. 19.1 Schematic representation of P-gp. The P-gp secondary structure, embedded in the cell membrane, is presented. P-gp possesses 12 transmembrane domains; and N-glycosylation, phosphorylation and ATP-binding sites are also indicated. The most common nonsynonymous polymorphisms, which induce encoded amino acid changes, and synonymous polymorphisms, which cause a silent mutation, are also shown.

P-gp interactions with its ligands, the direct structure-activity relationships (SAR) of P-gp remains to be clearly established. Better development of the SAR, which would increase the understanding of the pharmacological and physiological significance of P-gp, could eventually help in the prediction of drug entry into the brain through the BBB.

19.2.3 P-gp Substrates

The first molecules identified as P-gp substrates were generally from natural sources, either plants or microorganisms. Many drugs are transported by P-gp and their accumulation in the brain is limited (Table 19.1). Vinca alkaloids, epipodophyllotoxins, anthracyclines and taxanes are among the anticancer agents known to be transported by P-gp [39–41]. Increasingly, molecules other than anticancer agents have been identified as P-gp substrates. For example, P-gp trans-

Table 19.1 Substrates of P-gp.

Compound	Ref.
Anticancer agents	
Actinomycin D	178
Anthracyclines	181
Colchicine	183
Daunorubicin	178
Dexamethasone	82, 188, 189
Docetaxel	190
Doxorubicin	192, 193
Etoposide	194
Mitomycin C	178
Paclitaxel (taxol)	178
Tamoxifen	199–201
Vinblastine	178, 203
Vincristine	178
Immunosuppressive agents	
Cyclosporine A	57, 204, 205
Rapamycin	207, 208
Sirolimus	211
Tacrolimus	212
Others	
Opioids (morphine)	219, 220
Erythromycin (antibiotic)	222, 223
Okadaic acid	225
Steroids	44, 227
Aldosterone	44, 228
Cortisol	23, 44
Corticosterone	229
Glucocorticoids	44, 230
Progesterone	44
Sphingomyelin	231
Lovastatin (lipid-lowering agent)	226
Ca^{2+} channel blockers	
Diltiazem	179, 180
Mibefradil	182
Verapamil	184–187
Fluorescent dyes	
Rhodamine 123	191
Hoechst 33342	35, 36
Calcein-AM	195, 196
Tetracycline	197
Tetraphenylphosphonium	198
Ramosetron	202
HIV protease inhibitors	
Amprenavir	206
Indinavir	209, 210
Saquinavir	209
Ritonavir	213
Bioactive peptides	
Adrenorphin	48
Endomorphin 1 and 2	48
Somatostatin	49, 218
β-Amyloid	87
Cardiac drugs	
Digoxin	214, 215
Quinidine	178, 216
Digitoxin	217
Substance P	49
Toxic peptides	
Valinomycin	221
Gramicidin D	224
Cytokines	
Interferon-γ	45, 46
Interleukin-2 and -4	47

ports cardiac drugs, Ca^{2+} channel blockers, HIV protease inhibitors, immunosuppressive agents, fluorescent dyes and cyclic and linear peptides [40–43]. In addition to xenobiotics, various endogenous substrates for P-gp have been identified in normal tissues, including several steroids such as cortisol, corticosterone, progesterone and aldosterone [23, 44]. Also, cytokines (IL-2, IL-4, IFN-γ) and bilirubin have also been shown to be transported by P-gp [45–47]. Recently, the transporter has been shown to have high affinity for endogenous bioactive peptides, such as adrenorphin, endomorphin 1 and 2, somatostatin and substance P [48, 49].

Studies have shown that molecular weight (MW), surface area, aromaticity, amphiphilicity, proton basicity and H-bond accepting are important in determining P-gp substrate specificity [50–52]. Recently, it was proposed that P-gp substrate specificity could be approximated by three rules obtained from the MW, the H-accepting capacity (given by the Abraham's β coefficient) and from the ionization which is represented by the acid and base pK_a values of compounds. Thus, compounds with an Abraham's β coefficient ≥ 8 (approximately the total number of N and O atoms), MW >400, and $pK_a > 4$ are likely to be P-gp substrates, whereas compounds with $(N+O) \leq 4$, MW <400 and $pK_a < 8$ are likely to be non-substrates [53]. The application of this model could be useful in absorption, distribution, metabolism and excretion (ADME) profiling of new drugs. However, since P-gp possesses multiple binding sites and complex mechanisms for substrate recognition and transport, SAR models remain difficult to develop. The prediction of P-gp substrate specificity is influenced by several factors, including the types of assays used, the confusion between P-gp substrates and inhibitors and the binding possibilities with other targets such as cytochrome P450 3A4 [43, 53].

Reversal agents are molecules that restore sensitivity to anticancer agents in drug-resistant cancer cells by inhibiting the transport activity of P-gp. Three generations of these compounds have been used so far (Table 19.2). For example, calcium channel blockers, calmodulin antagonists, quinolins, steroids, immunosuppressive agents, antibiotics and detergents are reversal agents known in the first generation [54–56]. However, most of these agents produce significant toxicities when used at concentrations sufficient to inhibit P-gp. Several of these compounds are themselves substrates for P-gp and for other transporters. Among them, cyclosporin A (CsA) and verapamil were most often employed but cannot be used safely for MDR reversal at the dosage required. This led to the development of second-generation P-gp modulators, such as SDZ PSC 833 (valspodar), a CsA analogue [57]. Most of these agents have the same pharmacological properties as the original molecules but with less toxicity. In spite of their efficiency, many characteristics limit their clinical usefulness. It has been demonstrated that these compounds can significantly inhibit the metabolism and excretion of cytotoxic agents [58]. The high toxicity associated with this side-effect requires a reduction of chemotherapeutic doses in clinical studies [58]. Also, several second-generation P-gp modulators are themselves often substrates for cytochrome P450 3A4 enzyme or other transporter proteins such as

Table 19.2 Modulators of P-gp properties.

Modulator	Ref.	Medical use/analogy/type[a)]
First-generation compounds		
Cyclosporin A	57, 204, 205	Immunosuppressive
Nifedipine	232	Calcium channel blocker
Progesterone	233	Progestative
Quinidine	178, 216	Antiarrhythmic
Quinine	234, 235	Antimalarial
Tamoxifen	199, 200	Antioestrogen
Verapamil	184, 185	Calcium channel blocker
Second-generation compounds		
Valspodar (PSC833)	28, 236	Cyclosporin A
Cinchonine	234	Quinine
Dexniguldipine	237	Nifedipine
Dexverapamil	238, 239	Verapamil
Third-generation compounds		
Tariquidar (XR9576)	240	Anthranilamide
Zosuquidar (LY335979)	241, 242	Difluorocyclopropyldibenzosuberane
ONT-093	243	Substituted diarylimidazole
Tariquidar (XR9576)	244	Anthranilic acid derivative
Biricodar (VX710)	60, 245	Piperidine carboxylate
Elacridar (GF120918/GG918)	246	Acridone carboxamide
Natural compounds		
Curcumin	63	Polyphenol
Ginseng	85, 247, 248	Ginsenosides
Piperine	249, 250	Alkaloid (black pepper)
Catechins from green tea	66, 251	Polyphenols
Silymarin from milk thistle	252	Flavonoids
Garlic	253, 254	Organosulfur compounds

a) First-generation compounds: medical use. Second-generation compounds: analogy to first generation compound. Third-generation/natural compounds: type or chemical structure.

MRP1 [59, 60]. These compounds are in competition with the cytotoxic agent for transport by the pump, giving an unpredictable pharmacokinetic interaction. After disappointing results, a third generation of reversal agents was developed. These molecules aim to specifically inhibit P-gp function. These agents do not affect cytochrome P450 3A4 and were generally developed using SARs and combinatorial chemistry. Because they are noncompetitive inhibitors of the P-gp transporter, the use of this third generation of P-gp modulators permits a reduction in the dosage of chemotherapeutic agents.

The clinical efficacy of these reversal agents remains to be established, not only with regard to overcoming tumor resistance against chemotherapy, but also for other factors such as bypassing P-gp in the BBB. In recent years, several

new approaches have been developed to reduce and inhibit MDR1/P-gp expression in cells. Among them, the use of monoclonal antibodies or immunotoxins against P-gp, antisense oligonucleotides and small interfering RNAs (siRNAs) has been investigated [61, 62]. Furthermore, natural products from dietary intake, such as curcumin ginsenosides and piperine, have been identified as inhibitors of P-gp and several flavonoids, such as quercetin and naringenin, are reported to modulate P-gp activity (Table 19.2) [63–65]. Moreover, we have also demonstrated that epigallocatechin gallate, the major polyphenol present in green tea, inhibits P-gp activity [66]. At the same time, much effort has gone into investigating and identifying new natural compounds that inhibit P-gp, reverse the MDR phenotype and sensitize cancer cells to conventional chemotherapy without toxicological effects. However, other studies are necessary to understand the mechanisms involved in P-gp modulation by these natural products and to explore their potential in chemoprevention.

19.3 Localization and Transport Activity of P-gp in the CNS

19.3.1 Normal Brain

P-gp is found in many normal tissues with excretory function, including liver, kidney and small intestine [67, 68], and at blood-tissue barriers such as the BBB, blood-testis barrier and placenta [69]. As a result of its anatomical localization, P-gp is one of the most important transporters for drug disposition in the organism. It limits drug entry into the body after oral drug administration (enterocyte luminal membrane), it promotes drug elimination into bile and urine (hepatocyte canalicular membrane, kidney proximal tubule luminal cell membrane) and it limits drug penetration into sensitive tissues (brain, testis, fetal circulation).

The expression of P-gp in human BBB endothelial cells has been described in many studies, performed in various species (human, rat, mouse, cow, pig) [70–72] and indicates that the major site of BBB P-gp expression is at the luminal membrane of capillary endothelial cells (Fig. 19.2A, left panel). Several reports have shown that P-gp could be also present in the brain parenchyma. For example, in vitro P-gp expression and activity have been demonstrated in primary astrocyte rat brain cultures [73, 74] and in microglia [75]. In vivo, a recent study examined P-gp distribution, using confocal microscopy on rat brain sections and indicated that this transporter was preferentially expressed in the endothelial component but was also present in astroglial cells [76]. Another team observed that the P-gp pattern of expression in human and primate brain was the same as that seen for the astrocyte marker GFAP [77, 78]. Based on these immunofluorescent studies, a model of MDR in brain was proposed where P-gp is localized on astrocyte foot processes at the antiluminal side of the human BBB. More recently, this group published results where P-gp expression was found both in astrocytes and in endothelial cells of healthy primate brain [79]. In our laboratory, a study indicated that P-gp was strongly enriched in the positive endothelial cell fraction from brain and was absent from the negative fraction in which the glial fibrillary acidic protein (GFAP), an astrocyte marker, was present [80]. It was also shown by RT-PCR analysis that the *mdr1a* gene was preferentially expressed in this enriched EC fraction from the brain. At the subcellular level, our findings demonstrated that the P-gp

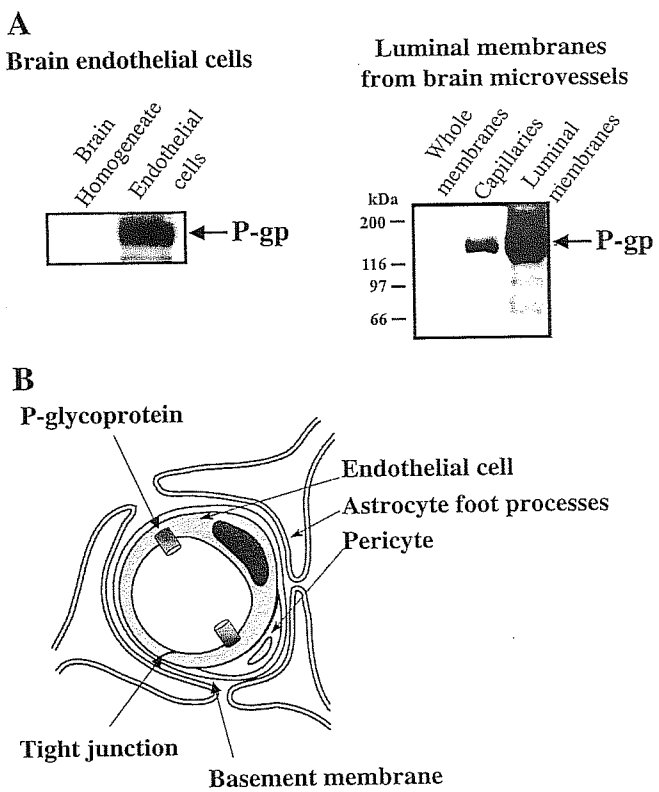

Fig. 19.2 P-gp expression at the BBB. (A) Left panel: Detection of P-gp in homogenates and in endothelial cells (EC) isolated from brain using magnetic cell-sorting beads. Right panel: Proteins from whole membranes, brain capillaries and endothelial luminal membranes were separated by SDS-PAGE and immunodetected with mAb P-gp (C219) antibody. (B) Schematic view of P-gp at the BBB. EC are sealed by continuous tight junctions and surrounded by a basal lamina. Pericytes are present at the periphery of vessels. Astrocyte foot processes are in close contact. P-gp is present in the luminal membranes of the brain vascular endothelium and impedes brain penetration by lipophilic substances.

was localized in isolated luminal membranes from the brain vascular endothelium in rat [81] (Fig. 19.2 A, right panel).

Overall, data obtained for most studies suggest that P-gp expressed in the capillary endothelial cells of the BBB restricts the CNS accumulation of many drugs, including chemotherapeutic agents (Fig. 19.2 B). This protective action of P-gp has been demonstrated using *mdra* knockout mice [82]. In addition to the expression of P-gp at the BBB, there have been a few reports of the expression and functional activity of P-gp in the choroid plexus [76, 83, 84]. P-gp was localized at the subapical side of choroid plexus epithelia and vectorial transport experiments performed on cultured rat choroid plexus showed an apically directed efflux function for P-gp, suggesting a role in preventing the export of certain substances out of the CSF, as opposed to its action at the BBB. More studies are needed to understand and characterize the role of P-gp at the choroid plexus.

19.3.2 Brain Diseases

The expression and activity of P-gp in the CNS plays an important role in the disposition and efficacy of pharmacological agents for brain diseases, such as brain tumors, epilepsy or HIV-associated dementia [85, 86]. In addition, P-gp also seems to play a key role in the etiology and pathogenesis of certain neurological disorders, such as Alzheimer's and Parkinson's diseases [87, 88].

In brain tumors, progress in clinical treatment has been slow and one of the major problems impeding treatment of these tumors is their weak response to anticancer drugs. In fact, brain tumors are known to develop MDR quite rapidly. Furthermore, gliomas are characterized by their infiltrative pattern of growth and it is likely that the blood-brain area in the tumoral periphery, which often escapes surgical intervention, possesses a totally functional BBB. The low response to chemotherapy may also depend on tumor blood flow, the integrity of the blood-tumor barrier and an inherent or acquired MDR phenotype in cancer cells [89]. As P-gp plays a major role in the defense of the organism against xenobiotics at the BBB [9], the determination of P-gp levels in brain tumors and peritumoral tissue is crucial for evaluating the long-term efficacy of chemotherapy. P-gp has been detected in endothelial cells from newly formed microvessels of gliomas [90–92], suggesting that, despite the leaky nature of the vasculature of gliomas, angiogenic vessels have maintained some of the restrictive capacities of the BBB.

19.3.2.1 Malignant Brain Tumors

We have reported that the P-gp expression levels detected by Western blot in various human malignant brain tumors (low-, high-grade gliomas) are similar to the levels of P-gp expression found in normal brain [93]. This is in agreement with previous studies which reported the presence of P-gp in resistant and partially chemosensitive glioblastomas by immunohistochemistry, using the monoclonal antibody C219 [94–96]. These results suggest that the poor response of brain tumors to many anticancer drugs may be related to the presence of this efflux transporter in cell populations of the primary brain tumors and that P-gp may be considered as a negative factor when predicting the outcome for patients with brain tumors. These findings also suggest that P-gp expression is maintained in both low- and high-grade gliomas. Moreover, the widespread expression of P-gp in these tumors may reflect an intrinsic resistance to anticancer drugs.

Previous immunohistochemical analyses showed that most gliomas and, more specifically, endothelial cells within the gliomas, stained positively for MDR1 P-gp [91, 92]. These studies support the concept that clinical drug resistance may be caused by P-gp expression, not only in cancer cells but also in the capillary endothelial cells of brain tumors. The role of the BBB in the low efficacy of chemotherapy is still unclear. Alterations in the brain capillary ultrastructure have been described, leading to an increase in the microvascular permeability in gliomas. In contrast, it has been reported that the neovasculature of even high-grade tumors preserves partial BBB permeability properties at the cellular level [97] and that the BBB at the tumor periphery is still intact. In addition, a study indicated that P-gp, one of the best phenotypic markers of the BBB, is expressed at the same levels in all primary tumors as in normal brain, indicating that brain tumors retain an important characteristic of the BBB which restricts the brain uptake of chemotherapeutic agents. Thus the BBB, especially at the edge of tumors, remains a formidable obstacle for drug distribution to brain regions that have been infiltrated by neoplastic cells [98].

19.3.2.2 Brain Metastases

Brain metastases occur in 20–40% of cancer patients and the estimated incidence in the United States is 170 000 new cases per year [99]. Lung cancer (9.7–64.0%), breast cancer (2–25%) and melanoma (4–20%) are the most common primary sources of metastases to the CNS [100]. Strikingly, we found that brain metastases from melanomas and lung adenocarcinomas exhibit only 5% and 40%, respectively, of the P-gp levels found in normal brain [93]. Metastatic malignant melanomas are recognized for their poor response to chemotherapy, whereas some effects of chemotherapy have been observed for lung adenocarcinomas [101]. The low expression of P-gp in these brain metastases suggests that MDR mechanisms other than P-gp could be responsible for their poor response to chemotherapy. The lack of P-gp expression in primary lung tumors and corresponding brain metastases also indicates that these brain metastases do not acquire the levels of P-gp expression found in normal brain tissue.

19.3.3
Expression of Other ABC Transporters at the BBB

It has been reported that efflux transporters other than P-gp are also expressed in brain capillaries. For instance, members of the MDR-associated protein (MRP) family have been detected at the BBB. In humans, seven MRP homologues have been identified [102]. All members of the MRP family are distributed throughout most human tissues [103]. MRP1, which was first described in 1992, was immunodetected by Western blots in human and rat choroid plexus, but the presence of MRP1 in the EC of brain capillaries remains controversial [104]. In animal models, Western blot and RT-PCR analysis suggest that MRP1 is expressed in isolated rat brain capillaries, primary cultured rat, pig and cow BCECs and immortalized rodent BCECs [105–107]. However, in isolated human brain capillaries, no expression of MRP1 was observed by immunohistochemistry [108]. The canalicular multispecific organic anion transporter (cMOAT or MRP2) was principally detected in hepatocytes, intestine and kidney but was not detected in endothelial cells of rat brain capillaries by Western blot [109]. Recently, MRP1, -4, -5 and -6 were shown to be expressed in primary BCECs by RT-PCR analysis as well as in a capillary-enriched brain extract [110]. In addition, MRP mRNA levels appeared to be closely associated with resistance to etoposide, adriamycin and vincristine in human glioma cell lines derived from patients [111]. Recently, levels of MDR1 and MRP1–MRP4 mRNA were compared between normal brain tissue and malignant gliomas [112]. The expression of both MDR1 and MRP2 were similar in normal brain and tumors, whereas MRP1 and MRP3 expression increased with tumor grade. Therefore, some of the MRPs may also confer intrinsic MDR activity in human gliomas or in metastatic brain tumors.

19.3.4
Subcellular Localization of P-gp

P-gp was also found in a specialized microdomain of plasma membranes called caveolae. This P-gp expressed in caveolae was first identified in multidrug-resistant cells [113–115], where it appears to play an important role in drug resistance development [116, 117]. Caveolae are flask-shaped plasma membrane invaginations involved in many cellular events such as transcytosis, endocytosis, cholesterol transport and signal transduction [118]. A family of proteins called caveolins comprise the structural component of caveolae. Caveolin-1 and caveolin-2 are primarily expressed in adipocytes, endothelial cells, smooth muscle cells and type I pneumocytes [119], whereas caveolin-3 is expressed in muscle and glial cells [120, 121]. Caveolin-1 possesses two isoforms (α, β) whereas three isoforms (α, β, γ) were reported for caveolin-2. MDR is a multifactorial process and recently an upregulation of caveolae and caveolar constituents, such as caveolin-1, -2 and glucosylceramide, was observed in different MDR cancer cells compared to their drug-sensitive counterparts [113, 114, 122, 123].

Localization of P-gp in caveolae has been also shown in the brain by different means. First, using a detergent-free method for caveolae isolation, our group showed the enrichment of P-gp, caveolin-1 and cholesterol in the low-density microdomains of human isolated brain capillaries and endothelial cells of an in vitro BBB model [115, 124]. Second, immunocytochemical analysis demonstrated the presence of P-gp in plasmalemmal vesicles of rat brain capillaries and in an immortalized rat brain endothelial cell line, RBE4 [125]. Then, Virgintino et al. [126] showed, by microscopy, that a large proportion of P-gp and caveolin-1 colocalize in the luminal compartment of the endothelial cells in human microvessels of the cerebral cortex. Besides endothelial cells, immunocytochemical analysis shows that, in astrocytes, a portion of P-gp is localized in caveolae [74] and colocalized with caveolin-1 [79].

In addition to the colocalization of some P-gp and caveolins in caveolae, our coimmunoprecipitation studies indicated that a population of P-gp molecules interacted with caveolin-1 in endothelial cells of the BBB [115, 124]. This coimmunoprecipitation was also reported in MDR cells [115, 127] and in astrocytes [74]. Similar to caveolin-1, caveolin-2 interacts with P-gp; and these three proteins form a high molecular mass complex at the BBB [124]. Oligomeric forms of P-gp have been observed in MDR cells and brain capillaries [28, 128] and recent data suggest that P-gp oligomerizes through indirect interactions [129]. The involvement of caveolins in P-gp oligomerization remains to be investigated, as well as the possibility of other proteins interacting with P-gp, like actin, ezrin, radixin, moesin, calnexin, Hsp70 and Hsp90 beta [130–132].

P-gp contains in its N-terminal portion a consensus caveolin-binding motif present in many proteins known to bind the scaffolding domain of caveolin-1 (Fig. 19.1). Three related caveolin-binding motifs are known ($\Phi X\Phi XXXX\Phi$, $\Phi X\Phi XXXX\Phi XX\Phi$, $\Phi X\Phi XXXX\Phi XX\Phi$, where Φ is a phenylalanine, tyrosine or tryptophan residue and X is any amino acid residue) [133]. The scaffolding domain of caveolin-1 regulates signaling molecules localized in caveolae such as eNOS, protein kinase C, insulin receptor, EGF and VEGF receptors [134, 135]. In the case of P-gp, mutation of its caveolin-binding motif decreases the interaction between P-gp and caveolin-1 and increases P-gp transport activity, indicating that caveolin-1 negatively regulates P-gp activity [124]. Moreover, overexpression of caveolin-1 in drug-resistant cells expressing P-gp causes a reduction of P-gp activity and in the cells become drug-sensitive, supporting the hypothesis that caveolin-1 inhibits drug transport by P-gp [127].

19.4 Polymorphisms of P-gp

19.4.1 MDR1 Polymorphisms at the BBB

In recent years, researchers have started to investigate the molecular mechanisms underlying inter-individual differences in the pharmacological effects of drugs. Genetic variations in drug transporters have received particular interest since they are among the factors determining the pharmacokinetic profile of drugs. Efforts have also been made to identify genetic variations of the human MDR1 (ABCB1).

Single nucleotide polymorphisms (SNPs) result in a single nucleotide substitution and possibly a change in the encoded amino acid. More than 40 SNPs and insertion/deletion polymorphisms in the ABCB1 gene have been reported. MDR1 gene SNPs are located in the coding region and in the noncoding region, including the core promoter region and the intron-exon boundaries [136–138]. The most common allelic combinations of MDR1 SNPs, which encode no amino acid changes, are synonymous polymorphisms at exon 12 (C1236T) and exon 26 (C3435T) and nonsynonymous polymorphisms (encoding amino acid changes) at exon 21 (G2677T). The localization of these major SNPs is shown in the schematic representation of P-gp (Fig. 19.1). Allele frequencies vary widely in MDR1 SNPs, particularly between populations of African descent and other ethnic groups. A large discrepancy is observed in these populations with an average of 18% and 48% frequencies, respectively, for the T allele of the three common allelic combinations of MDR1 SNPs (Table 19.3). Moreover, the segregation observed in the African American population consistently presents different, specific genetic combinations of MDR1 SNPs (haplotype) [136, 138, 139]. Consistent with wild-type allelic frequency in individuals of African origin, epidemiologic studies have observed a lower incidence of ulcerative colitis in Africans as compared with Caucasians [140]. It was suggested that the higher frequency of the wild-type allele (CC) in the African population for exon 26

Table 19.3 Most frequent allelic combinations of MDR1 genetic variations [136–138]. CA=Caucasian; AA=African American; AS=Asian American; ME=Mexican American; PA=Pacific Islander.

Exon	SNP	Allele frequency (%)				
		CA	AA	AS	ME	PA
12	C1236T	35–46	21	68	45	57
21	G2677T	42–46	10	45	40	36
21	G2677A	2–10	0.5	6–22	0	36
26	C3435T	48–56	20–23	40–49	50	50

(C3435C) SNPs may have resulted in a selective advantage against intestinal tract diseases [141]. There have also been no cases of neurotoxicity reported after treatment with ivermectin for the prevention of onchocerciasis in Africa, even though this drug causes neurotoxicity in animals with low P-gp expression [139, 142]. Thus, it has been demonstrated that some MDR1 polymorphisms have an impact on P-gp expression and function. The introduction of nucleotide changes in highly conserved regions of the MDR1 gene has a major impact on P-gp function and expression, in comparison with a nucleotide substitution introduced in less conserved regions of the gene [40, 143–145].

19.4.2 MDR1 Polymorphism and Brain Pathologies

At the BBB, impairment of P-gp function or altered P-gp expression level has been associated with severe neurotoxic side effects following administration of drugs or xenobiotics [146–148]. The SNPs in exon 26 (C3435TT or CT) genotypes are associated with low P-gp expression in the BBB in comparison to the CC genotypes references. A five-fold increased risk for developing Parkinson's disease was found in exon 26 (C3435T) heterozygous (T) and homozygous (TT) patients exposed to pesticides [88, 149]. Children with acute lymphoblastic leukemia (ALL) with the C3435TT or CT genotypes demonstrated a better response to chemotherapeutic drugs (e.g. etoposide, vincristine, doxorubicin), thus reducing the risk of CNS relapse [150]. Furthermore, the nonsynonymous G2677T SNPs in exon 21, combined with the synonymous C3435T SNPs in exon 26, increased the neurotoxicity of the immunosuppressive drug tacrolimus in liver transplant patients [151]. Patients with resistance to epileptic drugs have shown a higher frequency of the C3435CC genotype than the C3435TT SNPs. The well known C3435T polymorphism is silent (no amino acid changes) and raises the possibility that the polymorphism is not in itself causal but that different mutational groups (SNPs) forming different haplotypes in a consistent network are possibly the causal events [152]. Thus, linkage disequilibrium of the C3435T SNPs with other SNPs has underscored the importance of understanding haplotypes.

Interstudy comparison of the polymorphic effects on P-gp expression and function requires extensive haplotype analyses [136–138, 153, 154]. This will also provide a powerful tool for predicting and optimizing drug therapy, particularly for drugs with narrow therapeutic indices where induction or inhibition of transporter function can have a tremendous impact on drug efficacy and safety [136, 138, 155–157].

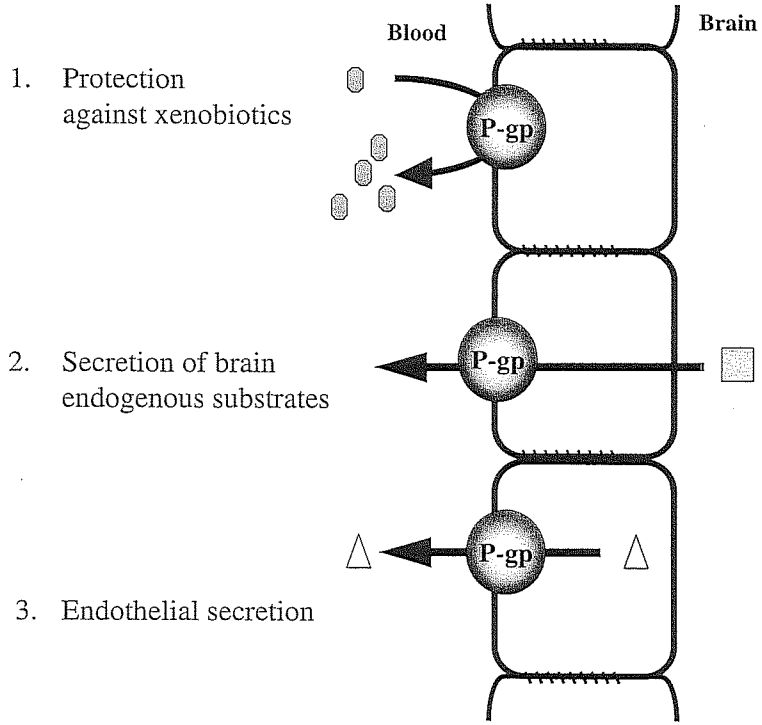

Fig. 19.3 Roles of P-gp at the BBB. P-gp at the BBB could have different physiological roles such as: (1) protection against xenobiotics, (2) secretion of brain endogenous substrates and (3) endothelial secretion.

19.5 Role of P-gp at the BBB

In brain capillaries, P-gp appears to play an important role in preventing many hydrophobic molecules from crossing the BBB and reaching the CNS. However, the exact physiological function of P-gp in the BBB is not completely understood. A growing body of evidence links P-gp to physiological roles distinct from its initially recognized function as a drug efflux system (Figs. 19.3 and 19.4).

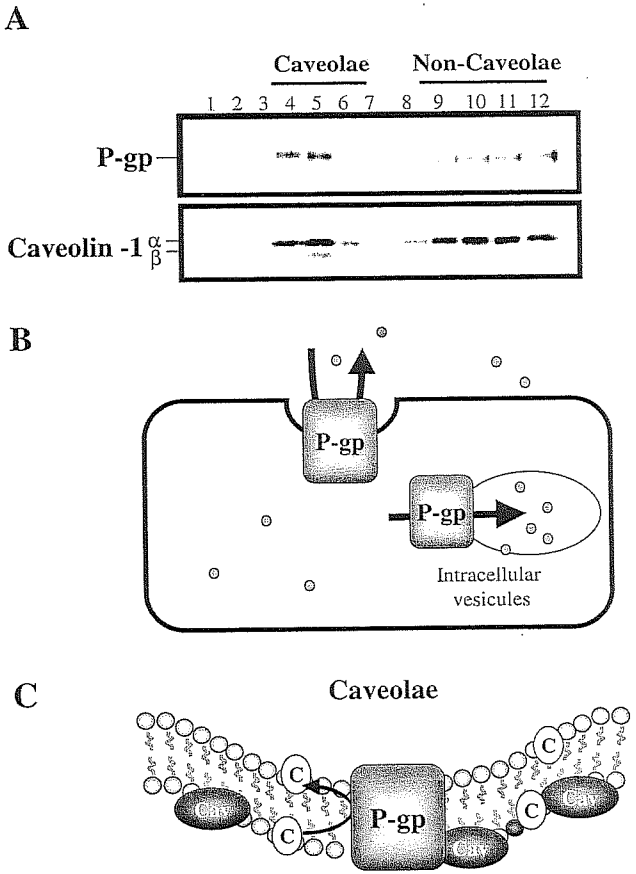

Fig. 19.4 Proposed roles of P-gp localized in caveolae.
(A) Low-density caveolae-enriched domains were isolated from bovine brain capillary endothelial cells (BBCEC) cocultured with astrocytes, using a carbonate-based fractionation method. Each fraction from a sucrose gradient was separated by SDS-PAGE and immunodetected using antibodies directed against P-gp (mAb C219) and caveolin.
(B) Since cholesterol is important for P-gp activity, the localization of P-gp in enriched cholesterol microdomains (caveolae) contributes to decreasing intracellular drug concentrations by pumping drugs inside caveolar vesicles and increasing their elimination outside the cells.
(C) P-gp mediates cholesterol redistribution from the cytosolic leaflet to the exoplasmic leaflet of the plasma membrane. C=Cholesterol; Cav=caveolin-1.

19.5.1
Protection Against Xenobiotics

Numerous reports provide functional evidence for P-gp-mediated drug efflux at the BBB. The interaction of drugs with P-gp in rat brain capillaries was demonstrated by photoaffinity labeling [158]. The generation of transgenic mice with a disruption of the *mdr1a* gene provided a pharmacological tool for the study of P-gp function in the BBB [9, 20]. These mice are viable, fertile and do not display obvious phenotypic abnormalities, indicating that this protein is not essential to their vital functions. However, P-gp substrates accumulate in the brains of these mice to a much greater extent than in wild-type animals and they are more sensitive to central neurotoxicity. For example, knockout mice are 50–100 times more sensitive to the neurotoxic effects of the pesticide ivermectin. The accumulation of this drug in brain tissue of *mdr1a*$^{(-/-)}$ mice was increased 80–100 times as compared to control mice. Recent application of in situ brain perfusion to wild-type and P-gp-deficient *mdr1a*$^{(-/-)}$ mice made it possible to assess the influence of P-gp on brain uptake of substrates without the potentially confounding differences in systemic pharmacokinetics upon P-gp distribution [159]. In summary, as indicated by Schinkel in 1999, P-gp appears to be a major efflux transporter at the BBB that acts as a guardian of the CNS by preventing the accumulation of many drugs in the brain [9].

19.5.2
Secretion of Endogenous Brain Substrates and Endothelial Secretion

In addition to its guardian role, P-gp is involved in the excretion of toxic compounds by renal proximal tubules and hepatic canalicular membranes [68, 69] and in the secretion of endogenous molecules from adrenal glands [160]. Thus, P-gp could fulfill a similar function in the BBB and be responsible for the secretion and/or excretion of brain-derived substances or metabolites into the blood (brain secretion). It could also be involved in the secretion of molecules from the endothelium itself (capillary secretion). In this respect, P-gp has been proposed to be involved in the release of neuroactive substances from the brain directly into the systemic blood following intracerebroventricular injection [161]. In addition, it has been demonstrated that β-amyloid (Aβ) is transported across the plasma membrane of P-gp-enriched vesicles in an ATP- and P-gp-dependent manner, suggesting that Aβ might be an endogeneous substrate for P-gp in brain [87]. Thus, a change in MDR1 function or expression might alter the clearance of Aβ from the brain and may contribute to cerebrovascular angiopathy. Since the accumulation of Aβ in the brain is a feature of Alzheimer's disease, the mechanism of Aβ transport opens new avenues in the understanding of Alzheimer's disease.

19.5.3
Caveolar Trafficking

Why P-gp is localized in caveolar microdomains remains to be established. However, several roles can be proposed for P-gp in these membrane microdomains, which are illustrated in Fig. 19.4. As mentioned, a portion of the P-gp localized in the brain capillary endothelial cells was found in caveolae microdomains (Fig. 19.4A). It has been proposed that the P-gp localized in the caveolae of MDR cells might act to decrease intracellular drug concentrations by pumping drugs inside caveolar vesicles and increasing their elimination (Fig. 19.4B). Different observations in MDR cells support this role of P-gp in caveolae: increase of caveolae and caveolar constituents in MDR cells compared to their drug-sensitive counterparts [113, 114, 116, 117, 123, 162], localization of P-gp in caveolae in MDR cells [113, 115] and drug sequestration in P-gp-containing cytoplasmic vesicles in MDR cells [163, 164].

Studies have also reported that a portion of the P-gp expressed at the BBB is colocalized with caveolin-1 in caveolae [125, 165]. Other studies have demonstrated that the P-gp expressed at the BBB can also interact with caveolin-1 [115, 124]. Considering the protective role of P-gp at the BBB in preventing the accumulation of many hydrophobic molecules and potentially toxic substances in the brain, modification of caveolae or caveolin levels might affect brain homeostasis. In this regard, a dramatic decrease in caveolin-1 expression has been observed in brain tumor endothelial cells compared to normal brain endothelial cells [166]. Since caveolin-1 inhibits P-gp activity [124, 127], a reduction in caveolin-1 expression could affect drug transport across the BBB and decrease chemotherapy efficiency.

In addition, studies from different groups have suggested the involvement of P-gp in lipid transport (Fig. 19.4C). Studies on P-gp activity, either drug binding or drug transport, in cells where P-gp is localized in caveolae or low-density microdomains show that P-gp is functional in these cholesterol-enriched microdomains [124, 167]. Moreover, studies have shown that cholesterol is important for the activity of P-gp, suggesting that caveolae might provide a favorable environment for its activity [168–170]. Specifically, cholesterol could interact with the substrate binding site of P-gp, suggesting that cholesterol may be transported by MDR1 P-gp (Fig. 19.4C) [171]. Furthermore, one study has shown that P-gp mediates the ATP-dependent relocation of cholesterol from the cytosolic leaflet to the exoplasmic leaflet of the plasma membrane, suggesting that P-gp might contribute to stabilizing caveolae [172]. It was also reported that caveolin-1 binds cholesterol and mediates its efflux within caveolae via an identified, cytosolic caveolin-1 complex comprising heat-shock protein 56, cyclophilin A and cyclophilin 40, which carries cholesterol to the plasma membrane caveolae [173]. In addition, P-gp in caveolae might contribute towards decreasing the formation of ceramide, which is involved in apoptosis induction [174]. Elevated levels of glucosylceramide, the precursor of ceramide, were observed in MDR cells and appeared to be due to the high activity of glucosylceramide synthase activity. Also,

it has been shown that sphingomyelin (SM) and the enzyme converting SM into ceramide, called sphingomyelinase, are enriched in caveolae in MDR cells [175, 176]. However, further studies are required to have a better understanding of the role of P-gp in caveolae at the BBB.

19.6 Conclusions

Overall, P-gp plays an important role in brain protection at the BBB. Its expression at the luminal side of endothelial cells in brain capillaries prevents the passage of many agents into the brain [9, 81, 177]. Moreover, many studies have proposed that the capillary EC of brain tumors participates in the resistance associated with P-gp expression, especially at the edge of tumors, where the BBB remains a formidable obstacle for the penetration of anticancer drugs into the brain regions infiltrated by cancer cells [98]. The development of P-gp inhibitors in order to reverse the MDR phenotype has been extensively investigated with generally disappointing results. The current, third-generation inhibitors present high potency and specificity for P-gp. Further studies are required to establish their contribution to potential therapeutic treatment by reversing P-gp-mediated MDR. It was recently reported, in MDR cells, that a portion of the P-gp present in the endothelial cells of the BBB is localized in caveolar microdomains [113, 115]. This particular localization could be useful for understanding the function and regulation of P-gp in drug elimination and transport across the BBB. Finally, recent observations have challenged the notion that P-gp has evolved merely to mediate the efflux of xenobiotics and raised the possibility that P-gp and related transporters might play a fundamental role in regulating cell differentiation, migration, proliferation and survival [174].

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