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Imaging the Function of P-Glycoprotein With Radiotracers: Pharmacokinetics and *In Vivo* Applications

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Abstract

P-glycoprotein (P-gp), an efflux transporter, controls the pharmacokinetics of various compounds under physiological conditions. P-gp-mediated drug efflux has been suggested as playing a role in various disorders, including multidrug-resistant cancer and medication-refractory epilepsy. However, P-gp inhibition has had, to date, little or no clinically significant effect in multidrug-resistant cancer. To enhance our understanding of its *in vivo* function under pathophysiological conditions, substrates of P-gp have been radiolabeled and imaged using single-photon emission computed tomography (SPECT) and positron emission tomography (PET). To accurately quantify P-gp function, a radiolabeled P-gp substrate should be selective for P-gp, produce a large signal after P-gp blockade, and generate few radiometabolites that enter the target tissue. Furthermore, quantification of P-gp function via imaging requires pharmacological inhibition of P-gp, which requires knowledge of P-gp density at the target site. By meeting these criteria, imaging can elucidate the function of P-gp in various disorders and improve the efficacy of treatments.

Transporters belonging to the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily maintain chemical homeostasis by mediating the transport of molecules across a membrane irrespective of concentration gradient. The ABC transporters are encoded by 48 genes in the human genome and have been grouped into seven subfamilies (designated ABCA–ABCG) based on sequence homology.[1,2] These genes encode membrane proteins with a range of subcellular localizations and substrate specificities. Of these transporter genes, ABCB1 has been most studied because it encodes P-glycoprotein ((P-gp) or multidrug resistance 1 (MDR1)), a 170-kDa lipoprotein widely expressed in plasma cell membranes of healthy human tissues and multidrug-resistant tumors.[1,2]

STRUCTURAL AND FUNCTIONAL CHARACTERISTICS OF P-GP

The structural and functional characteristics of P-gp help explain its role under both physiological and pathophyiological conditions.[3] Structurally, the transporter consists of two

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interwoven transmembrane regions, each containing six transmembrane helices and an ATP-binding site located intracellularly (Figure 1 and refs. [1,4,5]). The transmembrane helices of P-gp allow it to bind and induce efflux of a broad range of substrates with varying affinities. Functionally, P-gp regulates the transport of biologically important molecules, nutrients, hormones, and xenobiotics into and/or out of cells.[3] Although substrates for P-gp tend to be hydrophobic or weak base molecules with a planar ring system,[6] P-gp is considered polyspecific because it can recognize a wide range of substrates, including antiarrhythmics, antihistamines, cholesterol-lowering statins, and HIV protease inhibitors.[1] A number of detailed models have been proposed for the mechanism of substrate efflux, and it is generally agreed that ATP hydrolysis initiates substrate extrusion (Figure 1 and refs. [3,4]). By regulating the intra- and extracellular concentration of molecules, P-gp helps maintain chemical homeostasis.

Distribution and function of P-gp under physiological conditions

P-gp is widely expressed in the normal human body and plays both excretory and protective roles (Figure 2). Localization and pharmacokinetic studies have shown that P-gp can pump substrates out of tissue into the luminal space, ultimately excreting substrates out of the body. To function in this excretory role, P-gp is widely expressed in the cell membranes of organs such as the kidney, liver, and intestines.[2,5,7] In the kidney, P-gp localizes to the brush border of the proximal tubules, excreting substrates into the urine. In the liver, P-gp is localized to the apical membrane of hepatocytes, where it transports substrates into the bile. In the intestines, P-gp localizes to the apical membranes of the mucosal cells in the lower gastrointestinal tract, where it transports substrates to be eliminated in feces.[2,5]

In order to function in a protective role, P-gp is expressed in the membranes that create an interface between two organs (Figure 2). At these interfaces, such as the blood–brain barrier (BBB), the blood–testes barrier, and the placenta, P-gp protects crucial organs (i.e., brain, testes, and fetus) against the entry of xenobiotics.[1,2] To protect the brain, P-gp localizes on the apical (blood or lumen) side of endothelial cells[1,2] and on astrocytes whose foot processes line the BBB.[6] These two locations allow the transporter to prevent the penetration of toxic hydrophobic substances into neural tissue. Loperamide, for example, is an opiate that does not cross the BBB under normal circumstances, but on P-gp inhibition or disruption, it enters the brain and causes respiratory distress typical of opiates.[6,8] At the blood–testes barrier, P-gp localizes to endothelial cells and transports substrates into the lumen of surrounding blood capillaries.[1,2] Finally, to protect the fetus, P-gp distributes to the apical surface of the placenta and prevents the entry of foreign substrates.[1] P-gp has also been found on the apical (ventricle-facing) surface of the epithelium of the choroid plexus, at the blood–cerebrospinal fluid barrier,[2] but its role here is unclear because drugs do not appear to enter the cerebrospinal fluid by this route.[9]

Definitions of terms: substrate, inhibitor, and competitive substrate

In the context of transporters, *substrates* are compounds that are transported, whereas *inhibitors* are compounds that restrict the function of the transporter.[3,10] In the literature, the term *inhibitor* is often used synonymously with *modulator*, and the concepts are not clear-cut. For example, the compound verapamil is a substrate for P-gp at low concentrations, but, like many substrates, verapamil also inhibits ATP hydrolysis at high concentrations.[3] Inhibitors, such as cyclosporin A and tariquidar, interfere with ATP hydrolysis at all concentrations.[3,11] In this article, we use *substrate* to describe a drug transported by P-gp, *inhibitor* to describe a drug that restricts P-gp function, and *competitive substrate* to describe a drug that acts as both substrate and inhibitor, by partially inhibiting or antagonizing P-gp as a competitive substrate.

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IMAGING P-GP FUNCTION *in ViVo* USING RADIOLABELED SUBSTRATES

Two noninvasive imaging techniques—single-photon emission computed tomography (SPECT) and positron emission tomography (PET)—are regularly used to visualize and measure *in vivo* processes with great sensitivity. These techniques typically quantify the pharmacokinetics of a radiolabeled drug that is injected at tracer (subpharmacological) doses and that binds with high affinity to a specific receptor target. Through modeling, pharmacokinetic parameters are then used to determine the so-called binding potential of the receptor site; the binding potential is the product of receptor density and the affinity of the radioligand for the receptor.[12] In the case of P-gp, however, radiolabeled binding agents (e.g., inhibitors) have not yet been developed for *in vivo* imaging, although they do exist for *in vitro* studies—e.g., [^{3}H]tariquidar.[11] Instead, radiolabeled substrates measure *in vivo* function rather than the binding potential of this efflux transporter. In other words, P-gp function is implied by the absence of substrate in a protected organ rather than the presence of substrate binding to the transporter.

Imaging methods to measure P-gp function

For quantifying the *in vivo* function of transporters, three methods have been reported. The first, the *blood efflux index method*, measures the efflux rate of intracranially administered drug across the BBB. For example, Kakee *et al.* determined the efflux rate of [^{3}H]3-O-methyl-D-glucose from brain to blood in rats.[13] However, the administration route renders testing other tissue sites clinically impractical and prevents whole-body use.

The second method, the *metabolite extrusion method*, uses a prodrug/drug approach to image transporter function. A radiolabeled prodrug is developed that will readily cross the BBB when injected intravenously. On entering brain tissue, the prodrug is metabolized to a radiolabeled drug that is a substrate for the desired transporter. Because only the drug is actively pumped across the BBB, one can measure the efflux rate of the drug without measuring influx rate, eliminating the need for arterial sampling. This method, however, requires that the pro-drug convert completely to drug within a short time frame, so that the radioactivity measured is from the drug only. Okamura *et al.* used this method to image the function of the ABC transporter ABCC1 (multidrug-resistance protein, or MRP1) in wild-type and ABCC1-knockout mice using 6-bromo-7-[^{11}C] methyl purine.[14] Synthesis of prodrug and identification of a transporter-specific substrate may be limiting factors for this method, but this novel method has great potential in SPECT and PET. Furthermore, prodrugs could be developed for which metabolism would occur only in specific tissue regions.

The third and most common method involves the use of a radiolabeled P-gp substrate to measure the difference in uptake in the target tissue before and after inhibition of the transporter. Under baseline conditions, the protected organ or tumor accumulates minimal amounts of the radiolabeled substrate. After P-gp inhibition, the organ accumulates far more substrate.[15,16] In a tissue in which P-gp is absent or dysfunctional, the uptake of substrate should be unaffected by inhibition.

The function of P-gp can be quantified *in vivo* using standard compartmental models, but their application is complicated by P-gp's location directly on the border between two compartments. Using a simple configuration of two compartments (blood and tissue), k_1 is the rate of influx and k_2 is the rate of efflux (Figure 3). For a protected organ like the brain, does P-gp increase the rate of efflux or decrease the rate of influx? The answer depends on the site of drug administration and the mechanism of P-gp itself. If the drug is administered intracerebrally (as described earlier), P-gp clearly increases k_2 by transporting substrates from brain to blood. However, if the drug is administered intravenously, its entry into the brain is blocked, and this would be measured as a decrease in k_1.[16] Although this distinction may seem

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academic, it does reflect the putative mechanism of P-gp to transport molecules while in the lipid bilayer (Figure 1); that is, the substrate is "captured" and transported before it enters the intracellular compartment. Therefore, if P-gp captures all of the substrate while in transit through the membrane, its effect is entirely on k_1.[16–19] However, if some of the substrate escapes and has time to interact with the intracellular milieu, and if there is an efflux from the cell later, P-gp will both decrease k_1 and increase k_2. These theoretical considerations are limited, of course, by the temporal sensitivity of the imaging device and the rapidity of the *in vivo* rate constants. The uptake of [^{11}C]verapamil and [^{11}C]-*N-desmethyl*-loperamide ([^{11}C] dLop) in the brain have been carefully studied with this compartmental modeling approach.[16–19] Both radiotracers are such avid substrates for P-gp that almost all of the effect of P-gp is to decrease k_1, i.e., to block the entry of the radiotracer to the intracellular compartment.[16–19]

Considerations for pharmacological inhibition of P-gp

The density of P-gp present on the surface of the tissue significantly affects the dose of inhibitor needed to deliver more substrate to the tissue. Kalvass and Pollack used kinetic modeling to show that the concentration of the inhibitor tariquidar necessary to increase substrate in the brain by 50% is 100 times greater than the inhibition constant (K_i) of tariquidar.[20] In other words, >80% of P-gp expressed at the BBB must be inhibited to increase the concentration of loperamide in the brain by 50%.[20] Analogous to the concept of "spare receptors," this effect can be understood from the perspective of "spare transporters." That is, significant passage of the substrate occurs only after a sizeable percentage (e.g., 80%) of the transporters are blocked.

P-gp expression varies among organs; therefore, when an inhibitor is systemically administered, the phenomenon of spare receptors will result in differential effects on the passage of substrates to tissues. Choo and colleagues measured the effective dose of inhibitor that elicits 50% of the maximum substrate signal (ED_{50}) in three P-gp-containing tissues/organs in mice: T lymphocytes, testes, and brain.[21] Using loperamide as the P-gp substrate and tariquidar as the inhibitor, they found that the ED_{50} of tariquidar was lowest for T lymphocytes (ED50 = 0.3 mg/kg) and highest for brain (ED50 = 5.7 mg/kg). The 20-fold difference was attributable to the varying density of P-gp expression.[21] This finding held true in humans: coadministration of loperamide with 2 mg/kg tariquidar did not elicit the central nervous system effects of loperamide despite full inhibition of P-gp in lymphocytes.[8] In sum, the varying densities of P-gp in different organs affect the inhibitor dose necessary to elicit an increase in substrate signal.

SELECTION OF OPTIMAL SUBSTRATE RADIOLIGANDS OF P-GP

Although imaging of P-gp function is a relatively new area of research, three criteria have emerged for the selection of an optimal radiolabeled substrate: selectivity for P-gp, magnitude of the signal, and radiochemical purity of the signal. The selectivity of the ligand as a substrate for only P-gp is critical because of the varying expression of a number of transporters in the body and the common occurrence of cross-recognition of substrates. For example, the brain expresses P-gp (ABCB1), ABCG2, and several transporters of the ABCC family, most of which are apically (blood) directed at the BBB.[6] A candidate radioligand can be assessed to ensure that it is exclusively a substrate of P-gp, by using a range of *in vitro* assays[10] that are briefly described later.

Madin-Darby canine kidney and colorectal adenocarcinoma (Caco-2) cells have been engineered to overexpress a range of human and mammalian ABC transporters. These cells are grown as a polarized epithelial layer on Transwell inserts and form tight junctions that prevent paracellular diffusion of compounds; a given molecule can therefore traverse the well by passive diffusion or transporter-mediated processes.[7,10] A compound is identified as a

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substrate for an ABC transporter if it is transported from the basolateral to the apical side, and if transport in the reverse direction is prevented. The selectivity of a compound for P-gp can thereby be assessed, using cells expressing various ABC transporters. In addition, the selectivity of a substrate can be assessed by measuring its intracellular accumulation in parent cells as compared to that in ABC transporter–expressing cells. This accumulation assay is arguably easier to perform than that with polarized cells, and it has proven to be a good indicator of transport selectivity. For both assays, selectivity can also be confirmed by using selective inhibitors to block transport or accumulation. Other biophysical assays are available to further probe drug–transporter interactions; these include ATPase assays in vesicles, substrate competition assays, and time-course kinetics of transport.[10] In addition, these assays have been used to screen candidate radioligands for *in vivo* imaging. As Zhou *et al.* have demonstrated, drug candidates selected from *in vitro* assays can be tested *in vivo* in knockout mice.[9]

A second criterion for an optimal radiolabeled substrate for P-gp is the magnitude of the signal —namely, the ratio of the amount of substrate in the tissue at baseline to the amount after P-gp blockade. Because *in vivo* imaging has limited anatomic resolution, the visual effects in small organs become blurred or spill into their surroundings, an effect called the "partial volume error." Furthermore, variations between species and tissues in the expression of P-gp should be taken into account when assessing the suitability of the radiotracer. The magnitude of the signal in most *in vitro* assays of a substrate is therefore diminished when measured *in vivo*.

A third criterion for a radiolabeled substrate is the radio-chemical purity of the signal.[22] SPECT and PET measure total radioactivity and cannot distinguish radioligands from radio-metabolites. If the radiometabolite that is not a substrate for P-gp enters the tissue, it will increase the baseline signal and thereby blunt the relative magnitude of the signal after P-gp blockade. The presence of radiometabolites can be measured in animal models (e.g., P-gp knockout and wild-type mice), but the generation of radiometabolites in animals does not always reflect the situation in humans. When radiometabolism occurs, the substrate can sometimes be redesigned to avoid a particular metabolic route. For example, [^{11}C]loperamide generated significant concentrations of radiometabolites, including [^{11}C]dLop, in the brains of P-gp double-knockout mice (*mdr1a/1b*$^{-/-}$). However, injection of [^{11}C]dLop itself generated minimal additional radiometabolites in the brain (see later text and ref. [22]).

The stereospecificity of a radiotracer also contributes to its radiochemical purity, because stereoisomers can have differences in pharmacological properties such as metabolism or binding affinity for a receptor.[23] The PET radiotracer [^{11}C]verapamil is often used in its racemic form, as both (*R*) and (*S*) enantiomers are substrates for P-gp.[24] However, the metabolism of (*R*)-[^{11}C] verapamil is slower than that of its enantiomer (*S*)-[^{11}C]verapamil, and such differences in metabolism can compromise the quantification of kinetic parameters.[23] It is therefore desirable to use a pure stereoisomer if it retains substrate specificity and has little to no metabolism.

RADIOLIGANDS COMMONLY USED TO IMAGE P-GP FUNCTION

[^{99m}Tc]Sestamibi

The SPECT agent [^{99m}Tc]methoxyisobutylisonitrile ([^{99m}Tc]sestamibi) is a positively charged lipophilic complex (Figure 4) that readily enters cells in the absence of P-gp. It accumulates reversibly within mitochondria, which have electronegative membrane potentials.[25] Originally developed as an analog of K^+ for imaging myocardial ischemia, [^{99m}Tc]sestamibi was found to reflect P-gp function in Chinese hamster cell lines expressing varying levels of P-gp. In these studies, the intracellular concentrations of [^{99m}Tc]sestamibi decreased as P-gp expression increased, and [^{99m}Tc]sestamibi uptake by cells was enhanced in a dose-dependent manner by P-gp reversal agents such as verapamil and cyclosporin A.[25] Following this *in vitro*

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observation, P-gp function was imaged *in vivo* in athymic mice implanted with human tumor xenografts.[25] In humans, [^{99m}Tc]sestamibi was reported to image P-gp efflux transport in various MDR cancers.[25,26]

Although [^{99m}Tc]sestamibi was used as an *in vivo* marker of P-gp function, it is not the ideal substrate radioligand. For one, it has substrate activity for both P-gp and ABCC1 (Table 1 and ref. [25]), decreasing its utility to image the function of only P-gp. Furthermore, the magnitude of signal produced by [^{99m}Tc]sestamibi is smaller than those produced by other substrate radioligands. For example, the uptake of [^{99m}Tc]sestamibi in the brain, as determined by *ex vivo* measurements, was only fourfold higher in P-gp knockout mice than in wild-type mice (Table 1 and ref. [25]). [^{99m}Tc]Sestamibi is known to produce a radiochemically pure signal,[27] although its metabolism in humans has not been reported (Table 1). Consequently, of the three criteria for a substrate radioligand described earlier, [^{99m}Tc]sestamibi fulfills one (radiochemical purity) and marginally fulfills a second (magnitude of signal).

After the initial success with the SPECT tracer [^{99m}Tc]sestamibi, other radioligands were developed for PET: [^{11}C] colchicine, [^{11}C]verapamil, [^{11}C]daunorubicin, [^{11}C]paclitaxel, and [^{11}C]loperamide.[25,28] Although all of these were evaluated in animals, only two were extended to application in humans: [^{11}C]verapamil and [^{11}C]loperamide.

[^{11}C]Verapamil

[^{11}C]Verapamil (Figure 4), a calcium channel blocker, is both a substrate and an inhibitor of P-gp (ref. [29] and references therein). At the low concentrations used in PET, [^{11}C]verapamil functions as only a substrate.[3,24,30] The brain uptake of [^{11}C] verapamil in P-gp-knockout mice was 10-fold higher than that in wild-type mice as assessed by *ex vivo* measurements (Table 1 and refs. [29,30]). Many animal and human PET studies have been conducted with [^{11}C] verapamil, and these also showed increased uptake after P-gp blockade.[19,29]

[^{11}C]Verapamil has been extensively used to measure P-gp function, but this radioligand too does not fulfill all three criteria for a substrate radioligand (Table 1). Although [^{11}C]verapamil is a substrate for ABCB1, it has been reported to modulate the activity of ABCC1.[2] The radioligand does, however, fulfill the second criterion in that it produces a large-magnitude signal in the absence of P-gp (described earlier). Unfortunately, in humans, [^{11}C]verapamil undergoes extensive oxidative metabolism by cytochrome P450 enzymes. These radiometabolites of [^{11}C]verapamil, some of which have substrate activity for P-gp (refs. [17,23] and references therein), significantly limit its ability to quantify P-gp function.

[^{11}C]L operamide and [^{11}C]dLop

Loperamide is a potent over-the-counter opiate used to treat diarrhea, but it lacks effects in the central nervous system because it is an avid substrate for P-gp at the BBB. Investigators at GlaxoSmithKline were the first to use [^{11}C]loperamide (Figure 4) to monitor P-gp function *in vivo*, both in animals and in humans.[28] A major metabolite of [^{11}C]loperamide in animals and humans is [^{11}C]dLop, which we found to be also a substrate for P-gp.[22] Injection of the metabolite [^{11}C]dLop (Figure 4), rather than the parent compound, [^{11}C]loperamide, markedly decreases concentration levels of other radiometabolites that enter the brain.[15]

[^{11}C]dLop has a high signal strength and a pure radiochemical signal, thereby fulfilling two of the three criteria for an ideal substrate radioligand, but its selectivity for P-gp has not been reported (Table 1). First, with regard to selectivity, brain uptake was markedly enhanced in P-gp knockout mice and monkeys after blockade by inhibitors selective for P-gp (Figure 5 and refs. [15,16]). Although these results suggest selectivity for P-gp, systematic *in vitro* assays with various ABC transporters would be more conclusive. Second, with regard to magnitude of

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signal, brain uptake of [^{11}C]dLop by *ex vivo* measurements was 17-fold higher in P-gp-knockout mice than in wild-type mice.[15] As determined by PET imaging, the magnitude of this signal was only fourfold higher; the magnitude was blunted by the limited anatomic resolution of PET.[15] Finally, with regard to signal purity, [^{11}C]dLop produces minimal (<10%) radiometabolite concentrations in P-gp knockout mouse brain, as measured *ex vivo* at 30 min.[18]

UTILITY OF IMAGING IN CLINICAL TRIALS FOR THE REVERSAL OF P-GP-MEDIATED MDR

One of the clinical challenges in treating cancer is MDR. Resistance to chemotherapy has been observed since the first trials, and was first described in cell culture models in 1970.[5,31] Chinese hamster ovary cells grown in the presence of the chemotherapy drug actinomycin D were shown to be cross-resistant to several chemotherapy agents;[5] this resistance is ultimately linked to the increased efflux of chemotherapy agents by P-gp. Although genetic alterations such as defective apoptosis pathways are also known to confer resistance to cytotoxics, P-gp commonly confers the MDR phenotype to cancer cells.[5,32] The transporter can cause the efflux of a large percentage of known anticancer agents, most of which are derived from natural products, such as colchicine, daunorubicin, doxorubicin, etoposide, Taxol, vinblastine, and vincristine.[5,7] Therefore the expression of P-gp in response to one drug renders a cell cross-resistant to a broad range of other drugs.

Human cancers that develop in organs in which P-gp is normally expressed (gastrointestinal tract, liver, kidney, testes, and adrenal gland) are, as a result of expression of P-gp and other detoxifying mechanisms, intrinsically resistant.[1,2] Other cancers, such as acute myeloid leukemia, breast carcinoma, and non-Hodgkin's lymphoma, show associations between response to therapy and P-gp expression acquired during treatment,[1,2] suggesting a major role for P-gp in mediating resistance. Given that studies have linked increased expression of P-gp to impaired chemotherapy response[5] and lower patient survival,[2,32] a diagnostic probe to measure the *in vivo* function of P-gp is likely to be useful to assess its role in MDR cancer and, potentially, to aid in more individualized, targeted treatment.

Results from clinical trials involving P-gp reversal agents to reverse MDR

Many clinical trials have attempted to overcome the MDR phenomenon through pharmacological inhibition of the transporter.[31] The rationale for using P-gp inhibitors *in vivo* was based on early *in vitro* observations that the Ca^{2+}-channel blocker verapamil and the immunosuppressant cyclosporin A re-sensitized cancer cells to chemotherapy agents.[31] Hoping that the same outcome would translate *in vivo*, researchers developed numerous P-gp inhibitors and tested them in cancer patients. Thirty years of inhibitor trials have yielded little success. However, given the reasons for the failure of most of these clinical trials (see later text) and the diagnostic utility of SPECT and PET, MDR reversal through P-gp inhibition may still be clinically achievable.

The first compounds tested in clinical trials—first-generation P-gp competitive substrates such as verapamil and cyclosporin A—demonstrated the ability to inhibit P-gp but caused severe side effects.[2,31,33] To enable observation of tumor re-sensitization to chemotherapy after P-gp inhibition, plasma concentrations of verapamil needed to be 2–6 μmol/l, concentrations at which verapamil exerts cardiotoxic effects in humans.[31] Similarly, a phase III trial that coadministered cyclosporin A with chemotherapy agents in patients with acute myeloid leukemia showed improved survival but severe toxicity at the doses required to inhibit P-gp.[31] The majority of other trials with verapamil and cyclosporin A showed no overall improvement in survival rate, irrespective of cancer type.[31,33]

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The promise of clinical trials with first-generation reversal agents spurred the development and clinical testing of two second-generation inhibitors: valspodar (PSC833) and biricodar (VX-710). These new compounds showed more potent P-gp inhibition *in vitro* than the first-generation competitive substrates, and the pharmacological activities of their parent compounds were abolished. Yet they too produced disappointing results. Only one of five phase III clinical trials demonstrated improvement in survival rate when valspodar was coadministered with chemotherapy in patients with acute myeloid leukemia.[2,31,33] The other four trials found no benefit from administering valspodar, and toxicity issues persisted.[31] Other phase I/II trials with valspodar required dose reductions of the coadministered chemotherapy agent(s), presumably because of toxicity resulting from inhibition of other ABC transporters and detoxifying mechanisms, as well as from inhibition of P-gp. Trials with biricodar produced similar results, and the inhibitor did not enter phase III trials.[2,31,33]

The largely negative results from trials using second-generation inhibitors propelled the development and testing in the clinic of the current third-generation inhibitors that are much more P-gp specific. Results from clinical trials are limited, and most have not demonstrated clinically achievable P-gp inhibition in MDR cancer. One of the most tested third-generation inhibitors is tariquidar (XR9576). In phase I/II studies, tariquidar did not induce adverse toxicities, and one phase I study showed that 13 of 25 patients with refractory solid tumors experienced disease stabilization when tariquidar was coadministered with chemotherapy.[32] Yet phase III trials in non-small-cell lung cancer and breast cancer were terminated early either because patients receiving tariquidar experienced toxicities or because early data predicted little benefit.[32] Nevertheless, researchers are still investigating the utility of tariquidar in reversing MDR cancer, and several trials are still active.[32,33]

Another third-generation inhibitor that has demonstrated some success in targeting MDR cancer is zosuquidar (LY335979). Phase I trials in acute myeloid leukemia and in non-Hodgkin's lymphoma reported increased responses when zosuquidar was coadministered with chemotherapy.[31–33] Studies also showed that zosuquidar did not alter the pharmacokinetics of the chemotherapy agents[31] but did induce mild forms of ataxia and cerebellar dysfunction.[32] Even though many of these trials showed promising findings, the first phase III trial, conducted in 442 patients with acute myeloid lymphoma, found no benefit in overall survival when zosuquidar was added to the treatment, presumably because of the development of non-P-gp mechanisms of MDR.[31] More research is needed before treatment with zosuquidar can be deemed either beneficial or futile.

Reasons for negative results from cancer clinical trials of P-gp inhibitors

Given the multitude of negative results from these clinical trials, it is difficult to conclude that there is any benefit in using P-gp inhibitors to reverse P-gp-mediated MDR cancer, but there are many explanations for the negative results from these trials. First, many of the competitive substrates/inhibitors tested in early trials had unfavorable pharmacological properties that led to dose-limiting toxicities. Verapamil and cyclosporin A were developed to treat other medical conditions; their ability to inhibit P-gp was discovered later.[31] Given their poor selectivity for P-gp over other pharmacological targets, reversal of MDR was not observed until doses that induced toxicity were administered.

Although second-generation inhibitors had greater selectivity for P-gp over other pharmacological targets, they altered the pharmacokinetics of the chemotherapy agents, thereby causing toxicity. For example, valspodar interfered with the clearance and metabolism of chemotherapy agents, leading to toxic plasma levels of anticancer agents.[2,31] Similarly, biricodar was found to interact with other ABC transporters known to help with metabolism and clearance, thereby increasing plasma levels of chemotherapy agents.[31] As a result, many of the clinical trials required dose reductions of the administered chemotherapy agents, and

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these reduced doses may have been insufficient to elicit tumor regression. The problem of inhibitors altering pharmacokinetics of anticancer agents was, however, largely resolved by third-generation inhibitors that were more P-gp specific than those of previous generations.[31]

The third major reason for negative results seen in clinical trials is poor study design regarding either dosing regimens or patient selection. The two major phase III trials of tariquidar conducted in patients with non-small-cell lung cancer used higher doses of chemotherapy than was recommended. In the first study, vinorelbine, an anticancer agent, was coadministered at 25 mg/m^2 with tariquidar, whereas a phase I trial had shown the maximum tolerated dose of vinorelbine combined with tariquidar to be 22.5 mg/m^2.[32] The second study used a paclitaxel dose of 200 mg/m^2 in combination with tariquidar, a dose that is higher than recommended by the Food and Drug Administration. As a result, these trials were terminated early because of toxicities observed in patients receiving tariquidar.[32]

Apart from poor choices in dosing regimens and target tumors, the prevalence of various genetic polymorphisms of P-gp may have influenced results (both negative and positive) in clinical trials. Some single-nucleotide polymorphisms and haplotypes (combinations of single-nucleotide polymorphisms) of the *MDR1* gene have been shown to alter P-gp expression and activity both *in vitro* and *in vivo*.[7,34] For example, patients with ovarian cancer who express the wild-type allele for P-gp had a mean progression-free survival of 19 months when treated with chemotherapy, whereas those expressing the G1199A polymorphism had a mean progression-free survival of only 2 months.[7] Other studies have shown that plasma levels of certain chemotherapy agents may be lower in people with another polymorphism—the C3435T allele—than in those with the wild-type allele.[7] erefore the influence of genetic polymorphisms on the pharmacokinetics of anticancer agents and of P-gp inhibitors must be examined in future participants in clinical trials.

Finally, many studies investigated the reversal of MDR in cancers whose major mode of resistance may not be P-gp-mediated. Promising clinical trials were generally conducted against acute myeloid leukemia, breast carcinoma, and non-Hodgkin's lymphoma, cancers that are known to express P-gp. Clinical trials that yielded negative results were often conducted against cancers for which P-gp expression is not generally detected, such as small-cell lung cancer and non-small-cell lung cancer. P-gp expression in lung cancer is relatively low but is higher for ABCC1 (MRP1; ref. [1]). In effect, clinical trials such as the phase III trials of tariquidar in non-small-cell lung cancer were seeking to measure how effective P-gp inhibitors were in tumors that did not necessarily express the pharmacological target. Furthermore, the trials did not assess P-gp expression in the tumors of trial candidates—not surprising given the invasive nature of biopsies.

Utility of SPECT and PET in clinical trials of P-gp inhibitors

To improve future clinical trials, imaging may be useful in selecting patients whose cancers express MDR primarily though a P-gp-mediated mechanism. This can be done using a radiolabeled substrate as a surrogate marker for the *in vivo* function of P-gp. SPECT scans with [^{99m}Tc]sestamibi have accurately predicted chemotherapy response in a range of cancers,[26,35] even though [^{99m}Tc]sestamibi is also a substrate for ABCC1.[25] Patients whose tumors showed uptake of [^{99m}Tc]sestamibi responded well to chemotherapy, whereas those whose tumors showed little or no uptake (indicative of MDR) did not respond well. For a proper assessment of P-gp levels in tumors, patients should undergo two scans with a P-gp radioligand: at baseline and after P-gp inhibition. Patients whose tumors show enhanced uptake of the radioligand following P-gp blockade would be suitable candidates for P-gp inhibitor trials. Two SPECT studies have shown the utility of this method with [^{99m}Tc]sestamibi following administration of tariquidar (Figure 6 and ref. [36]) or valspodar,[35] and one ongoing study uses [^{99m}Tc]sestamibi to monitor progress throughout the trial.[32] Measurement of pharmacokinetic

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parameters can also determine the extent of P-gp function. Del Vecchio *et al.* found that breast cancer tumors expressing high amounts of P-gp had a 2.7-fold higher efflux rate for [^{99m}Tc] sestamibi than tumors expressing little or no P-gp.[37] Other studies have also confirmed that the efflux rate of [^{99m}Tc] sestamibi can predict the degree of MDR.[26]

IMAGING P-GP FUNCTION AT THE BBB IN HUMANS

P-gp function at the BBB of healthy humans has been imaged and quantified using [^{11}C] verapamil and [^{11}C]dLop (Figure 5). Recent studies have shown, however, that imaging of P-gp function at the BBB in humans may need to take into consideration a subject's age and genetic polymorphisms of P-gp. PET studies using [^{11}C]verapamil found lower P-gp function in the brains of older volunteers than in those of younger ones,[38] thereby suggesting that P-gp function declines with increasing age. Furthermore, haplotypes of nucleotide polymorphisms at positions 1236, 2677, and 3435 of the *MDR1* gene have been shown to alter P-gp activity *in vivo* and to alter substrate specificity *in vitro*.[7,34] However, imaging studies with [^{11}C] verapamil showed that its pharmacokinetics were unaffected in healthy volunteers who expressed either the TTT or the CGC (wild-type) haplotype.[39,40] Future imaging studies can detect whether haplotypes alter the pharmacokinetics of other SPECT and PET radioligands.

In contrast to healthy humans, relatively few patients with neurological disorders have been imaged to measure P-gp function. *In vitro* and animal data suggest that P-gp may play a role in disorders such as medication-refractory epilepsy, Parkinson disease, and Alzheimer disease, the strongest evidence being in epilepsy. As described later in this article, additional *in vivo* data are needed to elucidate whether P-gp has a pathophysiological role in these disorders.

Medication-refractory epilepsy

Although several antiepileptic drugs are known to be weak substrates of P-gp, they are effective in reducing seizures in the majority of patients with epilepsy. However, P-gp may play a role in medication-refractory epilepsy.[41] Biopsy specimens from patients with medication-refractory epilepsy showed a 130–200% increase in P-gp expression in cells immediately surrounding the epileptogenic focus.[41] Furthermore, rat models of refractory epilepsy showed that the duration and frequency of seizures directly correlated with P-gp overexpression. Seizures are known to not only transiently disrupt the BBB but also induce the release of glutamate, both of which signal nearby tissues to upregulate P-gp expression.[42] The only imaging study conducted in humans did not, however, report higher P-gp function in the epileptogenic focus of patients with medication-refractory epilepsy.[24] Nevertheless, the design of this study may have been flawed by a "ceiling effect." The uptake of [^{11}C]verapamil may have already been maximally blocked by normal P-gp function. The radioligand, therefore, may not have been able to measure supramaximal blockade.

Genetic polymorphisms of P-gp may also play a role in medication-refractory epilepsy, but association studies have reported conflicting evidence. Although an initial study found that the C3435T polymorphism of the *MDR1* gene was associated with medication-refractory epilepsy, other genetic studies conducted with a larger sample size did not.[43] A genetic study that investigated a common haplotype of three polymorphisms, instead of a single-nucleotide polymorphism, found a positive association with medication-refractory epilepsy.[44] Additional imaging studies with a P-gp-specific radioligand are needed to investigate the role of P-gp and of its polymorphisms in medication-refractory epilepsy.

Parkinson disease

A neurodegenerative disease classically characterized by the loss of dopamine cells in the substantia nigra, Parkinson disease is a movement disorder that may result from the

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accumulation of environmental toxins and from genetic factors.[45,46] Genetic association studies have shown that toxins, known to be transported by P-gp *in vitro*, increase the risk of Parkinson disease in individuals who carry certain MDR1 polymorphisms.[45] Although MDR1 polymorphisms were not examined, a pilot PET study using [^{11}C]verapamil demonstrated that radioactivity uptake was higher in the midbrain and dorsal pons of patients with Parkinson disease than in those of healthy volunteers.[47] This initial finding was intriguing because the location of increased uptake (and therefore of decreased P-gp function) includes the dopamine neurons that degenerate in Parkinson disease. However, subsequent imaging studies with [^{11}C]verapamil conducted in larger patient samples did not replicate the initial results[45,46] and therefore do not support the hypothesis that impaired P-gp function contributes to the pathogenesis of Parkinson disease.

Alzheimer disease

P-gp may play a role in the development of Alzheimer disease, a form of dementia characterized in postmortem brains by senile plaques containing an insoluble form of amyloid-β (Aβ; refs. [48,49]). Although the deposition of Aβ occurs in normal aging, the process is accelerated in Alzheimer disease.[49] A postmortem study of brains from patients with Alzheimer disease showed that P-gp expression was significantly and inversely correlated to deposition of Aβ, thereby suggesting that P-gp might play a role in controlling Aβ deposition in the brain.[49] *In vitro* experiments showed that Aβ is a substrate for P-gp, and imaging studies with [^{125}I] $A\beta_{40}$ injected intracranially in wild-type and P-gp-knockout mice showed that knockout mice had greater Aβ deposition than wild-type mice.[48] Furthermore, inhibition of P-gp with tariquidar in wild-type mice increased Aβ deposition, thereby confirming that P-gp is one mode of transport of Aβ out of the brain.[48] Another imaging study in a squirrel monkey model of Alzheimer disease demonstrated that intravenously injected [^{125}I]$A\beta_{40}$ was eliminated from the brain at a slower rate in affected monkeys than in controls.[50] These animal studies indicate that P-gp function in Alzheimer disease can be studied *in vivo* in humans, using P-gp radioligands.

CONCLUSION

By measuring the pharmacokinetics of radiolabeled P-gp substrates or inhibitors, SPECT and PET radioligands can image and quantify P-gp function *in vivo*. To ensure that P-gp function is measured accurately, substrate radioligands must have high selectivity for P-gp, produce a large signal after P-gp blockade, and generate few radiometabolites that enter the target tissue. Furthermore, imaging studies that measure P-gp function must take into consideration the density of P-gp at the target tissue and the “spare” transporter phenomenon, because this affects decisions regarding doses of inhibitor to be administered. If these criteria are met, substrate radioligands can provide useful information regarding P-gp function. These two techniques may help to improve our understanding of drug distribution and can indicate whether P-gp-related MDR can be reversed by coadministration of a P-gp inhibitor and a chemotherapy agent. Individualized treatment could be provided to cancer patients because MDR levels in tumors could be determined before or during chemotherapy treatment. Furthermore, imaging could provide insight into the possible role of P-gp in neurological disorders such as medication-refractory epilepsy and Alzheimer’s disease. Finally, SPECT and PET can measure any potential relationships between genetic polymorphisms of P-gp and its function; this is important, given that polymorphisms can alter the specificities of substrates for P-gp, and consequently alter drug absorption, disposition, and elimination. Although current SPECT and PET research focuses on imaging P-gp, researchers are beginning to examine additional drug transporters from the ABC family.[14]

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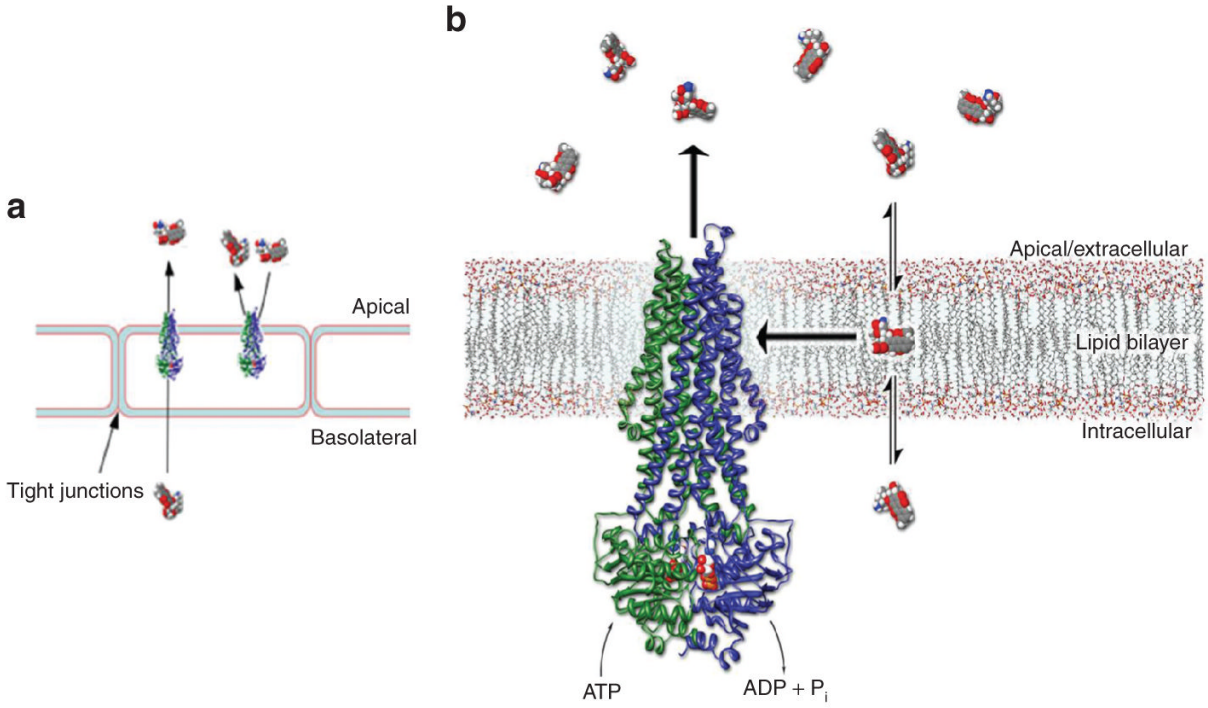

Figure 1.

Structural model of P-glycoprotein (P-gp) and a diagram of the mechanism by which it pumps substrates. (**a**) P-gp is a transmembrane protein located on the apical side of polarized cells that facilitates the translocation or prevents the ingress of molecules. Polarized cells are joined together by tight junctions that prevent paracellular diffusion and ensure that the passage of small molecules is transporter-regulated. (**b**) A model of P-gp in the lipid bilayer extruding doxorubicin (to scale). The binding and hydrolysis of ATP (shown bound during hydrolysis) initiate substrate extrusion. Substrates can be intercepted and extruded directly from the lipid bilayer or be drawn from the intracellular pool. The model of P-gp incorporated in the figure was kindly provided by Robert Rutledge.

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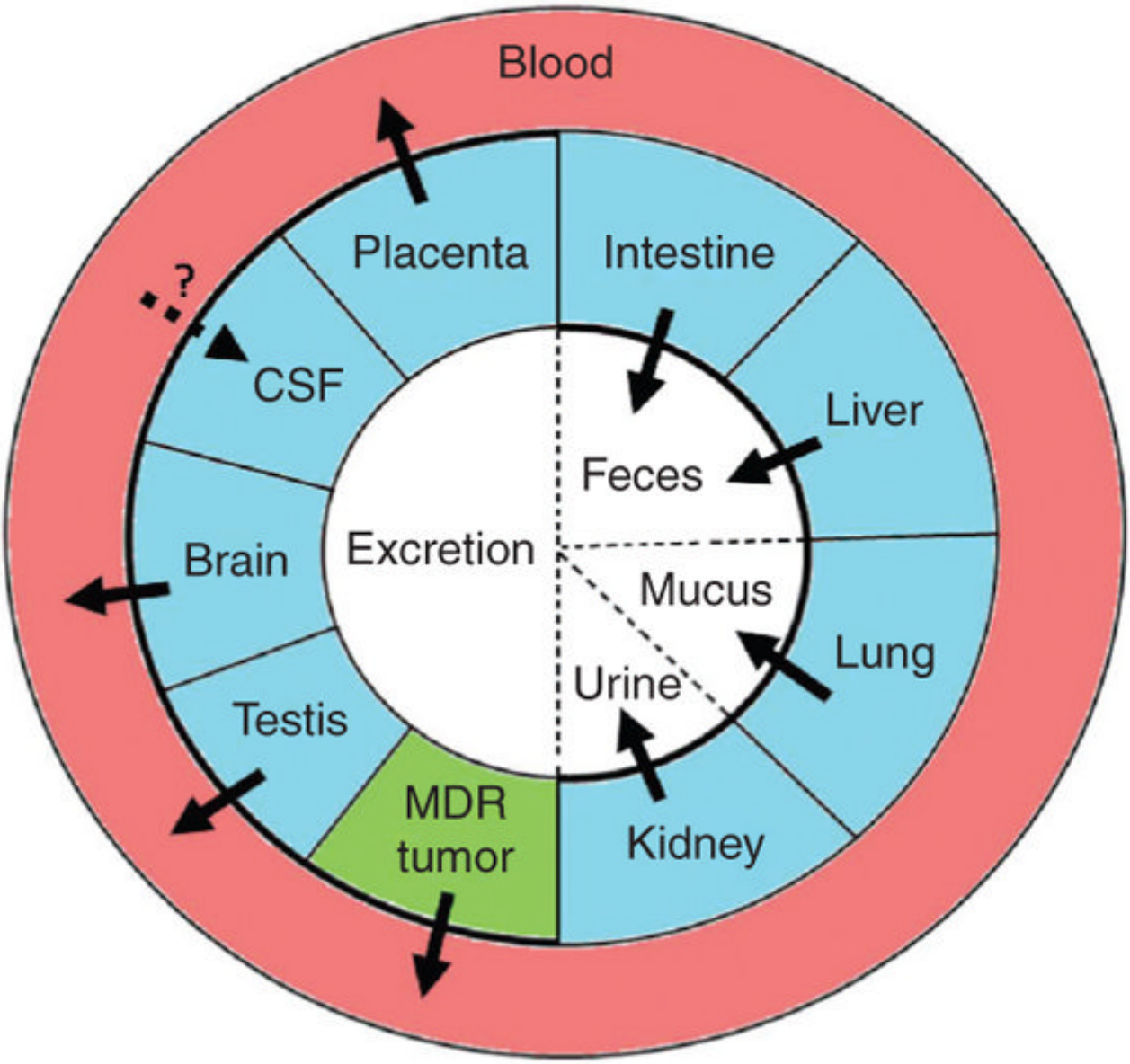

Figure 2.

Direction of substrate transport by P-glycoprotein (P-gp) located in various organs of the human body. The bold solid arrows indicate the known direction of transport, whereas the broken-line arrow indicates unclear direction of transport. P-gp is located in the lipid bilayer (thick black line) that forms a barrier between various organs; red indicates vasculature, blue represents tissue, and white indicates excreta. CSF, cerebrospinal fluid; MDR, multidrug resistance. Modified from ref. [2].

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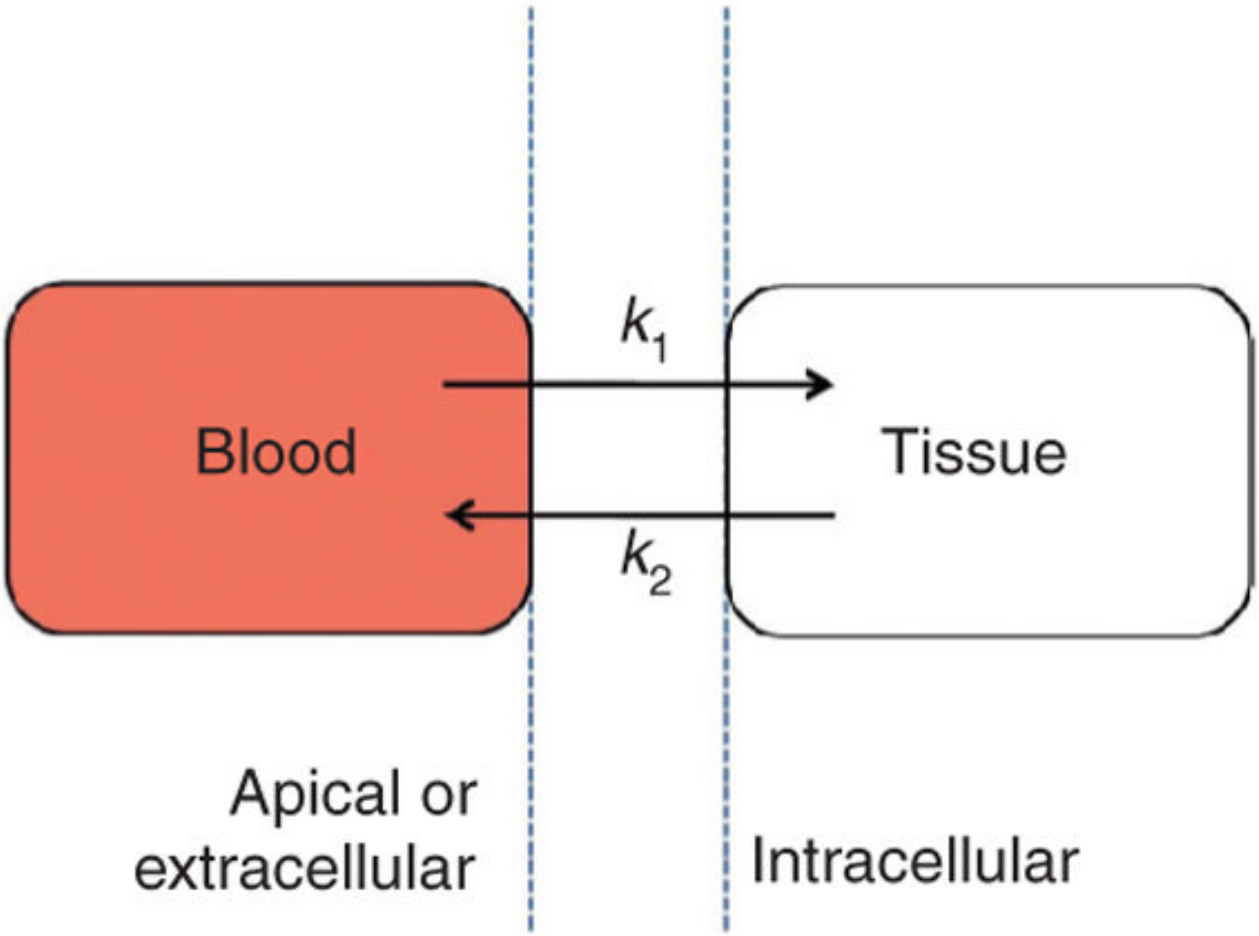

Figure 3.

Schematic representation of a simplified model of two compartments (blood and tissue) used to quantify P-glycoprotein (P-gp) function. The two compartments are separated by a lipid bilayer in which P-gp is located. The kinetic parameter k_1 (ml · cm^{-3} · min^{-1}) is the influx rate of the radiolabeled P-gp substrate, and k_2 (min^{-1}) is the efflux rate of the substrate from tissue to blood.

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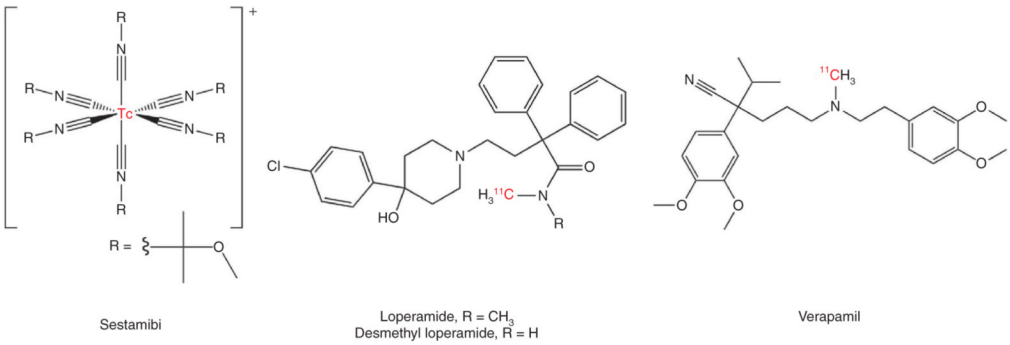

Figure 4.

Chemical structures of P-glycoprotein substrates radiolabeled for single-photon emission computed tomography and positron emission tomography imaging. The radiolabeled atom is indicated in red.

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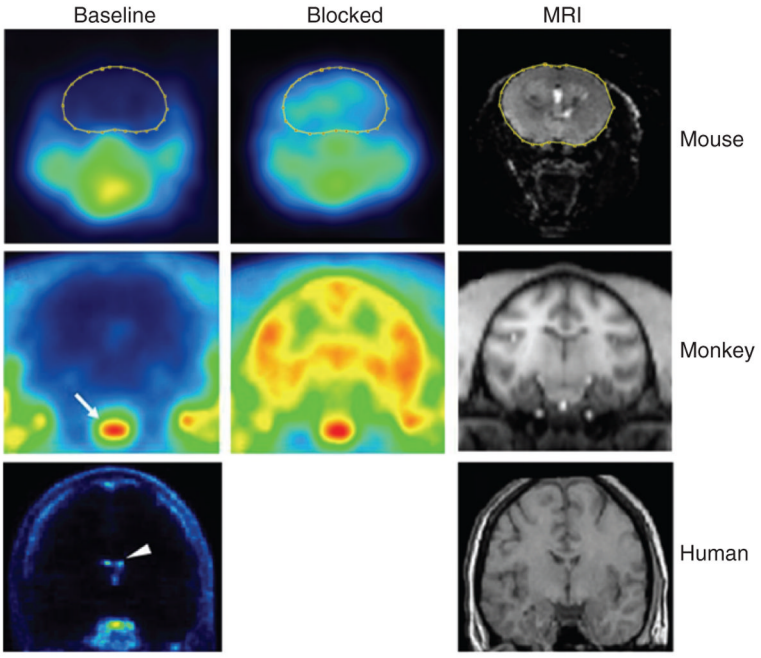

Figure 5.

Representative positron emission tomography (PET) and magnetic resonance imaging (MRI) images of brain after injection of [^{11}C]-*N-desmethyl*-loperamide ([^{11}C]dLop) under baseline and blockaded conditions in three species. Under baseline conditions, uptake of [^{11}C]dLop in the brain is minimal in all species (column 1), except in the pituitary in monkeys (arrow) and humans and in the choroid plexus (arrowhead) of humans. The choroid plexus shown in this section is located near the medial surface of the lateral ventricles and the roof of the third ventricle. A P-glycoprotein (P-gp)-knockout mouse brain (circled) shows higher uptake of [^{11}C]dLop than that of a wild-type mouse (row 1). Under P-gp-blocked conditions (column 2), after injection of the P-gp inhibitor DCPQ (8 mg/kg, intravenously (i.v.)) in monkey, the uptake of [^{11}C]dLop in the brain markedly increases (row 2). Preliminary studies in humans show a small increase (20%) in the uptake of [^{11}C]dLop in the brain after injection of tariquidar (2 mg/kg, i.v.). MRI images of corresponding coronal slices are shown in the column on the right. DCPQ, (2*R*)-anti-5-{3-[4-(10,11-dichloromethanodibenzo-suber-5-yl)piperazin-1-yl]-2-hydroxypropoxy}quinoline trihydrochloride. PET and MRI images for mice kindly provided by Jeih-San Liow; images for monkeys reprinted from ref. [16] with permission of the Society of Nuclear Medicine; and images for humans reprinted from ref. [18] with permission of the Society of Nuclear Medicine.

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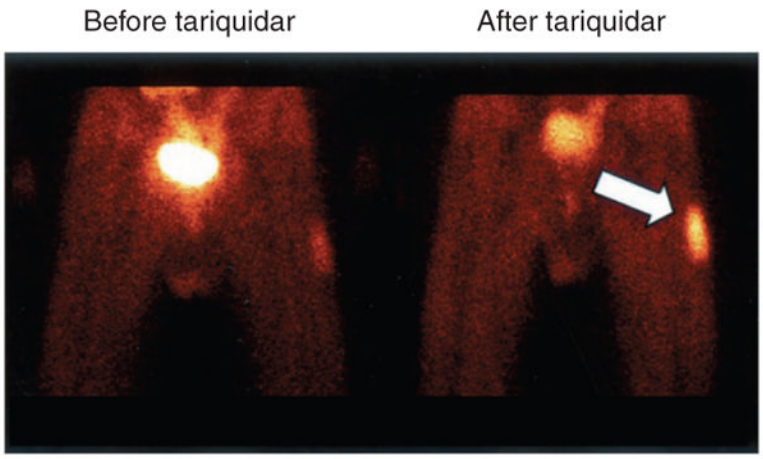

Figure 6.

P-glycoprotein (P-gp) inhibitor tariquidar (2 mg/kg, intravenously) increased the uptake of [^{99m}Tc]sestamibi in a metastatic tumor mass in the left thigh (arrow) of a patient with renal cell carcinoma. Bladder accumulation of [^{99m}Tc]sestamibi was lower after P-gp inhibition than at baseline, thereby supporting the hypothesis that P-gp excretes substrates into urine. Reprinted from ref. [36] with permission of the American Association for Cancer Research.

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Table 1

Evaluation of four radioligands in terms of three criteria for a good substrate radioligand

	Criteria for a substrate radioligand					
	Activity at ABC transporters in cultured cells				Radiochemical purity	
Radioligand	**ABCB1**	**ABCC1**	**ABCG2**	**Magnitude of signal in mouse brain**	**Mouse brain (%)**	**Rat brain (%)**
[^{99m}Tc]Sestamibi	Yes[25]	Yes[25]	No[27]	4[25]	NR	NR
[^{11}C]Verapamil	Yes[2]	Yes[2]	No[2]	10[30]	90[30]	70[51]
[^{11}C]Loperamide	Yes[22]	NR	NR	17[22]	50[22]	NR
[^{11}C]-*N-desmethyl*-loperamide	Yes[15]	NR	NR	16[15]	92[15]	NR

Values for magnitude of signal represent the ratio of parent radiotracer in brains of P-glycoprotein (P-gp)-knockout mice as compared to that in brains of wild-type mice, as determined by *ex vivo* measurements. Percentages of radiochemical purity represent composition of parent radiotracer in rodent brains at 30 min, with the exception of the value determined in P-gp-knockout mice at 60 min for [^{11}C]verapamil. Radiochemical purity of [^{99m}Tc]sestamibi is known to be high,[27] even though it has not been reported (NR) in humans.

The references cited are those from which data are derived.

ABC, adenosine triphosphate–binding cassette.