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P-Glycoprotein Recognition of Substrates and Circumvention through Rational Drug Design

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Abstract: It is now well recognized that membrane efflux transporters, especially P-glycoprotein (P-gp; *ABCB1*), play a role in determining the absorption, distribution, metabolism, excretion, and toxicology behaviors of some drugs and molecules in development. An investment in screening structure−activity relationship (SAR) is warranted in early discovery when exposure and/or target activity in an in vivo efficacy model is not achieved and P-gp efflux is identified as a rate-limiting factor. However, the amount of investment in SAR must be placed into perspective by assessing the risks associated with the intended therapeutic target, the potency and margin of safety of the compound, the intended patient population(s), and the market competition. The task of rationally designing a chemistry strategy for circumventing a limiting P-gp interaction can be daunting. The necessity of retaining biological potency and metabolic stability places restrictions on what can be done, and the factors for P-gp recognition of substrates are complicated and poorly understood. The parameters within the assays that affect overall pump efficiency or net efflux, such as passive diffusion, membrane partitioning, and molecular interaction between pump and substrate, should be understood when interpreting data sets associated with chemistry around a scaffold. No single, functional group alone is often the cause, but one group can accentuate the recognition points existing within a scaffold. This can be likened to a rheostat, rather than an on/off switch, where addition or removal of a key group can increase or decrease the pumping efficiency. The most practical approach to de-emphasize the limiting effects of P-gp on a particular scaffold is to increase passive diffusion. Efflux pumping efficiency may be overcome when passive diffusion is fast enough. Eliminating, or substituting with fewer, groups that solvate in water, or decreasing their hydrogen bonding capacity, and adding halogen groups can increase passive diffusion. Reducing molecular size, replacing electronegative atoms, blocking or masking H-bond donors with N-alkylation or bulky flanking groups, introducing constrained conformation, or by promoting intramolecular hydrogen bonds are all examples of steps to take. This review discusses our understanding of how P-gp recognizes and pumps compounds as substrates and describes cases where structural changes were made in a chemical scaffold to circumvent the effects of P-gp interactions.

Keywords: Active efflux; circumvention; drug resistance; drug membrane transport; P-glycoprotein; structure−activity relationship

1. Introduction

It is readily appreciated that the cost of bringing a successful pharmaceutical compound to market has become exorbitant. One reason for this is the challenge of discovering breakthrough drugs that possess all of the attributes that distinguish them as efficacious and safe. One way to defray these mounting costs is to create a more efficient process in discovering new chemical entities that can then be optimized as drug candidates with the intent of their becoming bona fide drugs. This is the essence of drug hunting rather than meeting a milestone of clinical candidate selection. Most

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pharmaceutical companies have integrated new processes, technologies, and decision tools, while strategically applying key organizational assets, to ensure that early discovery presents the best choices in targets and lead molecules.[1] A portion of the focus is pulling forward the risks associated with absorption, distribution, metabolism, excretion, and toxicology (ADMET) of new molecules by placing preclinical assays between the hit and lead or the lead and candidate selection stages. This is a daunting task as there are no single factors that remain constant as the chemical scaffold is strategically and iteratively altered to optimize pharmacological activity simultaneously with ADMET characteristics. All agree that our goal is to uncover defects that produce attrition as early as possible so that cost and time of development are effectively lowered.[2] The early use of this preclinical ADMET information concerning liabilities of the chemical starting point is expected to reduce downstream lead optimization cycle times and attrition rates.

One attribute within ADMET that has received considerable attention over the past decade is drug efflux transport.[3] Facilitated and active membrane transporters for specific nutrients have been recognized and studied for several decades, but most recently specific transporters that are capable of acting on a wide range of xenobiotics have been elucidated. There is a significant body of literature indicating that membrane efflux transporters can play a role in determining the ADMET behaviors of some drugs and molecules in development. Nearly all of the important mammalian efflux transporters are members of the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily where transport is driven by hydroylysis of ATP.[4] The first member of this family to be discovered was P-glycoprotein (P-gp; gene *ABCB1* or MDR1). It originally was identified as a key reason for multidrug resistance in treatment of certain cancers (reviewed by Gottesman et al.[5]); however, constitutive expression of P-gp in many normal tissues such as intestinal epithelia, hepatocytes, kidney proximal tubules, blood−brain barrier (BBB) endothelia, and placental trophoblast demonstrates its protective roles in limiting drug absorption, contributing to pharmacokinetics, and potentially impacting pharmacodynamics and toxicity.[6,7] Moreover, interactions of drugs with P-gp have received attention for facilitating or complicating drug−drug interactions and their role in adverse drug events.[8,9] It is not surprising then that P-gp is of particular interest to medicinal chemists and pharmaceutical scientists because of its ability to impact whether an optimal lead candidate is chosen to quickly become a drug. Some even consider P-gp interactions to be a serious liability to successful development of certain pharmaceutical agents.[10] As such, there is a plethora of reviews in the literature considering P-gp.[11−15] It also is important to point out that there are a variety of membrane transporters other than P-gp that are expressed within these biological barriers thus influencing both efflux and influx of drugs, and this may further complicate data interpretation and rational design.[7,15] It is well appreciated that there are overlapping substrate specificities between ABC transporters, and it is suggested that an overlap in substrate recognition between members of the solute carrier family (SLC), e.g., organic anion transporting polypeptides (OATP; *SLCO*), organic anion transporters (OAT; *SLC22A*), and ABC transporters, facilitates efficient vectorial transport across biological barriers such as the BBB.[16]

This review will attempt to collate our present understanding of the practical structure−activity relationship (SAR) of molecules as substrates for P-gp. Most other reviews focus on the activity of P-gp and its impact on particular drugs, including the use of P-gp antagonists to decrease these effects.[17−19] Few reviews,[20,21] or even primary papers, have focused on scaffold modifications that purposefully circumvent P-gp substrate interaction and liability. The paucity of examples, particularly outside of the cancer field, is surprising

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given the apparent attention that P-gp has garnered since the mid-1990s. One must believe that there are many successes and failures within the pharmaceutical industry when it comes to removing or introducing the propensity for a compound to be a substrate for P-gp. Although some debate remains around best practices, the right tools, or preclinical assays and predictive in silico methods, appear to be available for a rational structure−activity approach. This review discusses briefly our understanding of how P-gp recognizes compounds as substrates and describes cases where structural changes were made in a chemical scaffold to diminish the effects of P-gp interactions.

2. P-glycoprotein as a Drug Efflux Pump

Expression. P-gp is a 170-kDa, integral membrane protein belonging to the ATP-binding cassette (ABC) superfamily of transporters found in most organisms from bacteria to humans (reviewed by Ambudkar et al.[6]). It is only one of 48 known human members belonging to seven subfamilies (A−G),[4] so the potential for other transporters to effect drug ADMET is high.[15] Mammalian P-gp is a single polypeptide arranged as two homologous halves.[6] Each half contains six transmembrane domains (TMDs) and a hydrophilic region with an ATP-binding domain. Both halves are required for activity, albeit they do not have to be covalently linked, and both ATP-binding domains are required for activity. There is no X-ray crystallographic structure of mammalian P-gp; thus, our information about the P-gp pump three-dimensional structure is indirect and the subject of debate. Ambiguous protein structure homology models have been proposed based upon electron crystallography of mammalian P-gp and low resolution crystal structure of bacterial ABC transporters.[22,23] These models depict ABC efflux pumps as forming a ring with their 12 TMDs and the ATP binding domains positioned on the cytoplasmic side (Figure 1). This structural orientation as it relates to function is discussed more in the Mechanism of Action subsection below.

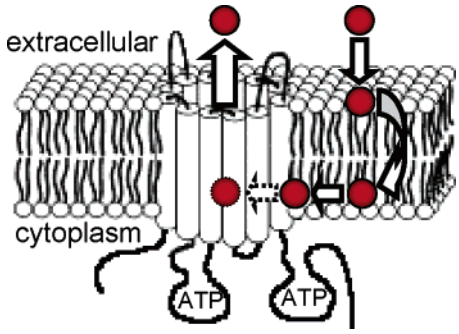

Figure 1. An idealized representation of P-glycoprotein and its 12 transmembrane domains (TMDs) within the lipid membrane bilayer. A proposed pumping process: substrate inserts into the bilayer interface, flips across the lipid core, accesses the TMD interface(s) from the inner leaflet, and is translocated to the central water-filled channel, where it is released to the extracellular space.

The expression and distribution of P-gp within tissues influences the ADMET of molecules.[8,24,25] This function is thought to protect vital tissues from the harmful effects of xenobiotics such as consumed plant alkaloids or metabolites. P-gp is found within lung, gut, liver, kidney, brain, testis, and placenta[15] (Figure 2). The efflux pump is expressed on only one membrane domain of a differentiated and functionally polarized cell type such that transport is asymmetric. In this way these cell types act as biochemical barriers by preventing access of blood-borne substrates to the underlying tissue or as facilitators to rid the underlying tissue of metabolites that are substrates.

Implications to Outcome. There is no doubt that P-gp efflux can have a profound effect on the pharmacokinetics and pharmacodynamics of therapeutic agents. Numerous reviews discuss preclinical and clinical studies demonstrating how P-gp reduces oral absorption, reduces brain exposure, or contributes to drug systemic elimination.[7,8,11−15,24−26] A researcher has the preclinical assays and tools available to identify a P-gp substrate and to demonstrate that the P-gp efflux activity can alter the disposition of a compound in vivo in preclinical species (see section 3). These studies are most likely to occur in rodent, and especially mouse, with the availability of P-gp-deficient mice.[27] It is less likely that

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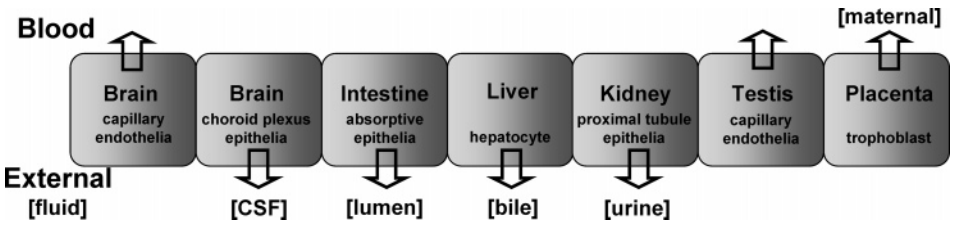

Figure 2. Tissue expression of P-glycoprotein and the net flux accomplished by its asymmetric location on the cell types identified to impact absorption or elimination.

a relevant demonstration of a pharmacodynamic effect will be made because of the inability to conduct an in vivo efficacy assay. Frequently, in vivo efficacy assays in the existing discovery flow schemes cannot be conducted in a P-gp-deficient mouse. The alternative approach, to create a chemical knockdown of P-gp in the in vivo efficacy assay species of choice using a P-gp inhibitor, is often difficult to do because it involves a codosing paradigm that might be complicated by unwanted drug−drug interactions or an insufficient response. Nevertheless, this approach has been taken to measure the magnitude of effect of P-gp on the efficacy of a test compound.[28−30] Yet, none of these approaches are able to size up the risk of taking the test compound/P-gp substrate into humans.[8] It is this aspect of taking the preclinical information forward as a prediction of what to expect in clinical studies that requires much more research.

It is therefore necessary to place into perspective the overall importance of what can be expected to occur if a compound is a P-gp substrate. This risk assessment should occur within the context of the intended therapeutic target, the potency and margin of safety of the compound, the intended patient population(s), and the market competition. We can make some generalizations based upon the literature and from experience. For example, the instances in early discovery where target activity in an in vivo efficacy model is not achieved, especially with confirmation of insufficient exposure, are cases where investment in screening SAR for P-gp interaction is warranted. More likely, most cases will involve a less obvious scenario where exposure is moderate to good and target activity is achieved. It is well understood that P-gp-mediated efflux of a test compound is in most cases more likely to have impact on exposure through a decrease in absorption rather than an increase in systemic elimination through bile or urine.[31] And, the importance of P-gp efflux to oral drug absorption is not likely to be quantitatively important unless it is in combination with low dose, low aqueous solubility, slow passive diffusion, and/or marked first-pass mucosal metabolism. Test compounds that are P-gp substrates are typically unaffected in their oral absorption as the P-gp is either saturated or overwhelmed.[11,31]

In contrast, test compounds with CNS targets are often quantitatively influenced by P-gp efflux because the exposing free concentrations in plasma are typically undersaturating to efflux pump activity.[31] The effect of P-gp efflux on brain uptake is not usually all or none. The quantitative importance of P-gp efflux on exposure and efficacy of a CNS-targeted test compound that is a P-gp substrate can vary depending upon target potency, P-gp efficiency, and target/test compound distribution. This concept of overall pump efficiency is described later as more than the traditional Michaelis−Menten definition of V_{max}/K_m. It is well established that many drugs including CNS active compounds are also P-gp substrates,[32,33] so simply identifying a hit as a P-gp substrate is not a "no go" criterion.

What then might be the risks to developing a lead molecule that is a P-gp substrate? One risk is an increased variability in interindividual exposure, which could result in greater variability in efficacy.[11,24] In addition to a potential for concentration-dependent exposure, especially within brain, there might be pharmacogenetic reasons.[34,35] Yet, for P-gp, the assuredness of mutation effects, despite the identification

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of polymorphisms, has not resulted in clear clinical importance.[24,36] Another risk is the potential for drug−drug interactions.[8,37] The pharmacokinetic/pharmacodynamic relationship and or toxicity of a candidate/P-gp substrate might be altered by another P-gp interacting compound, or vice versa. Generally, P-gp substrates/inhibitors are suspected of potential interactions with a drug like digoxin with a narrow therapeutic window and whose plasma levels are primarily influenced by transporter activities including P-gp.[8,34] This could lead to additional cost and time because of more clinical studies that might lead to an undesirable drug package insert.

Several recent reviews discuss the importance of regulated expression of P-gp within the different tissues.[9,38,39] While there is some evidence for widely varying functional differences in P-gp pumps within the various tissues, there are emerging data on induction of P-gp levels within tissues depending upon the compound studied. P-gp activity has been suggested in many cases to coordinate with cytochrome P450 (CYP) 3A4 activity, especially in the intestine, so it is not surprising that both may be similarly influenced by gene regulatory elements so that a maximal, and often transient, response can be achieved when increased protection is needed.[40−42] It also is possible that P-gp expression is induced under a pathological condition or following polypharmacological intervention for the same or a different indication.[43] Thus, the implications of induction of P-gp expression with regard to a test compound that is a substrate may be profound and should be considered with the risk of developing a P-gp substrate.

Mechanism of Action. A hallmark of P-gp is its apparent promiscuity in accepting a wide array of structurally diverse substrates. It clearly does not function like a traditional enzyme−substrate interaction as originally thought, and understanding the existing data for how a substrate accesses and binds the pump is key to appreciating its unique character. The P-gp pump is an ATPase requiring energy derived from the hydrolysis of ATP to actuate movement of a compound from the cell back out into the extracellular fluid (reviewed by Ambudkar et al.[6]). The exact mechanism used to accomplish this is not fully known, but accumulated data have led to proposed models. These data suggest that the process is multistepped[44] (Figure 1). First, substrate partitions into the outer leaflet of the lipid membrane bilayer. Subsequently the substrate must cross the membrane interior as the data indicate that the ligand binding site(s) of P-gp is accessible from the inner membrane leaflet.[45−47] This second step requires desolvation of substrate and reordering of the membrane lipids. Consequently, this flip-flop from outer interface to inner interface is considered rate limiting for eventual transport.[42,48,49] However, there are data indicating that certain ligands can bind P-gp directly from the exoplasmic leaflet of the lipid bilayer implying that flip-flop is not always required.[50] To complete transmembrane flux, the solute must be released from the opposite interface into solution, or into the cytoplasm. However, P-gp appears to bind substrate within the membrane, and most likely in the cytoplasmic leaflet of the lipid bilayer, described as the "vacuum cleaner" hypothesis.[45] This method of access leverages the two-dimensional, lateral diffusibility of partitioned compounds within the lipid membrane as a more efficient process than interactions randomly occurring after three-dimensional diffusion from the aqueous (extracellular or cytoplasmic) environment.[51] The events following these steps are even more vague and speculative. The P-gp pump appears to undergo a conformational change in response to ligand binding and ATP hydrolysis, which is proposed to

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transfer the bound ligand for exposure and release into the extracellular fluid. This might occur by a "flippase" activity that moves the ligand to the exoplasmic leaflet, or to the core of the ring that is thought to be formed by the TMDs (Figure 1), from where it is rehydrated and released into the extracellular fluid.[47,52,53] Data also support a two-step model for pumping of certain substrates (daunorubicin) where, after binding to a cytoplasmic-located site, compound is transferred to a second site on P-gp in the exoplasmic side of the membrane prior to release into the extracellular fluid.[50]

Drug Binding Sites. There are at least two asymmetric, yet interdependent (allosterically dependent), drug binding sites on P-gp.[54,55] These have been described as the H (for binding the ligand Hoechst 33342) and R (for binding the ligand Rhodamine 123) sites. Some compounds (vinblastine, actinomycin D) appear to bind both sites.[56] Each site is located near to the cytoplasmic leaflet of the lipid bilayer with the R site located slightly more shallowly or closer to the interfacial phospholipid headgroup region.[57,58] These sites are proposed to be located on the TMDs of P-gp with several studies identifying the locations to be at the interfaces between TMD 2 or 3 and TMD 11 and between TMD 5 and TMD 8.[23,47,53] These TMDs have recently been proposed to function as "gates" that open and close with the conformational change associated with pumping and resulting in "opened" and "closed" conformations of P-gp.[22,47] The conformational change is proposed to involve rotation of these TMD/TMD interfaces so that the bound ligand is moved from the membrane−protein interface to the water accessible channel.[53,59] It is unknown exactly how substrate is released, but rehydration of the ligand is proposed to facilitate this final step.[59] This concept appears to be consistent with the general mechanism proposed for other polyspecific drug efflux pumps such as bacterial lipid transporters.[22,23,53] It is not known which site corresponds to which interface. Moreover, the binding pocket could still be physically located in one general, but large, region on P-gp with the capacity to bind two ligands simultaneously, but occupying different molecular regions that could overlap.[58,59] This concept is consistent with some of the pharmacophore models that have been derived[55] (section 4). Different amino acids in these sites may substitute for one another to accommodate structurally diverse substrate molecules. Also, the plasticity of the TMD/TMD interfaces might contribute another fundamental postulate of multispecificity to substrate−pump interactions.[23]

The Importance of Membrane Interaction. No matter what the details of the pump mechanism of action, substrates most likely require physical−chemical attributes that allow them to interact with biological membranes.[60,61] The overall efficiency of P-gp-mediated transport will depend upon the affinity of substrate for transporter, the transport velocity, and the concentration of substrate within the immediate environment, which could be a specific subregion of the lipid bilayer. Others have pointed out the importance of considering membrane partitioning in modeling P-gp activity and predicting recognition.[10,49,62] Membranes are highly organized, anisotropic systems that are fluid enough to allow considerable translational, rotational, and flexing movements of the constituents.[63] Thus, rather large, charged and uncharged molecules can insert into the lipid−water interface, but only a fraction of charged molecules can permeate a lipid membrane. It has been proposed that the ability to partition into the membrane and modify surface tension could be a distinctive feature of P-gp ligands.[64] All P-gp ligands appear to be surface-active compounds, although the sampling size is small, and measured air−water partition coefficient can be used as a surrogate for membrane interaction.[49]

Thus, substrates must be lipophilic, or at least amphiphilic to gain access to the P-gp binding site. Cellular pharmacokinetic simulation showed that P-gp pumping efficiency is dependent on pumping capacity, affinity of the ligand−pump interaction, transmembrane movement rate, affinity of drug

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for target, and affinity of drug for nontarget, intracellular binding sites.[65] The effective concentration of the substrate in the membrane is then important for driving the interaction with the transporter, which is consistent with the vacuum cleaner model (reviewed by Hochman et al.[62]). Therefore, the efficacy of P-gp is similar whether it functions as a high- or a low-affinity pump.[65] Kinetic simulations suggest that P-gp will function effectively as a low-affinity pump extracting directly from the inner leaflet, whereas pumping from the outer leaflet appears futile.[65] One complicating and confusing observation has been that good substrates are sometimes poor inhibitors and good inhibitors are sometimes poor substrates.[62,66,67] We can rationalize this conundrum to the role of the membrane partitioning step. Good inhibitors might appear to be poor substrates because local concentration at the pump binding site might be high and saturate the pump. It also has been suggested that the ligand might bind the transporter so tightly that release is too slow, or that the removed molecule may immediately repartition in what has been described as a futile cycle analogous to bailing out a leaky canoe. Good substrates might appear to be poor inhibitors because they are unable to achieve efficacious local concentrations at the binding site. This also might be attributed to slow passive diffusion, or flip-flop, which can limit access to the binding site and, thus, prevent reaching local concentrations that are sufficiently above the Michaelis−Menten constant (K_m) to saturate the binding site.

Transport efficiency also will appear low for compounds with good intrinsic passive permeability, because residence time at the binding site will be short.[68] A long residence time in the membrane will increase the likelihood that P-gp will remove it. When the rate of transmembrane transport is fast enough, then pump cannot keep pace and will operate in a futile cycle[69] (reviewed by Eytan,[44] 2005). This effect was nicely demonstrated using a series of rhodamine analogues where transmembrane movement rate was inversely correlated with P-gp efficiency, and was not determined solely by the molecule's hydrophobicity.[70] Consequently, one circumvention strategy is to design compounds with rapid transmembrane movement[44] (section 4).

3. Assay Strategy

There are numerous in vitro assays available to screen compounds for interaction with P-gp. These assays have been reviewed elsewhere in detail.[62,66,71−73] Historically, many of the published structure−activity relationships were generated from concentration-dependent growth inhibition, or cytotoxicity, of cell lines with differing expression levels of P-gp.[10,73] This method has proven useful for anticancer compounds that are cytotoxic and demonstrate drug resistance. Improved activity in a resistant cell line can be interpreted as circumvention of P-gp assuming it is the only factor changing; however, this can be complicated by copresence of other drug resistance transporters or other reasons discussed in section 2. Another commonly used assay is the concentration-dependent effect of a compound on basal ATPase activity present in membrane vesicles from cells that overexpress P-gp.[74] This is an indirect measure of substrate transport and should be interpreted with caution since the validity of drug-induced ATP hydrolysis alone for differentiating substrates and inhibitors has been questioned.[62,66,71,72] Schwab et al.[72] recommended a screening strategy to aid in lead selection and optimization. They suggested that a higher-throughput P-gp inhibition assay involving cellular accumulation of the P-gp substrate calcein-AM (CAM) be used as a first-tier screen. Single concentrations at <50 μM could be tested at the lead identification stage. While capacity and throughput are undoubtedly high, the assay poorly distinguishes substrates and inhibitors of P-gp and is prone to many false negatives. Polli et al.[66] identified some P-gp ligands as false negatives in the ATPase and CAM assays mostly because of limiting, very slow passive permeability (see category IIB ligands below).

Both Polli et al.[66] and Hochman et al.[62] suggest that a direct measurement of transport is required for the most reliable identification of a P-gp substrate. Transport studies using adherent cell monolayers have been used to identify P-gp substrates and inhibitors. In these types of assays, perme-

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ability measurements in the apical-to-basal and basal-to-apical directions are made. The ratio of these numbers can then be used to provide some estimate about P-gp involvement. However, caution must be exercised when one is tempted to associate the magnitude of the ratio with potency, especially across unrelated structural scaffolds.[75] We choose Madin-Darby canine kidney (MDCK) epithelial cells that express the ABCB1 gene. Bidirectional transport is then measured using bioanalysis in the absence and presence of a specific P-gp inhibitor with this latter measurement giving the intrinsic passive diffusion for a compound. The method is more laborious and should be employed judiciously; however, we feel that it provides the best information to support structure−activity studies with the intent of circumventing P-gp. It can be streamlined for increased capacity and throughput by measuring flux over a single time interval and by cassette dosing if care is taken to anticipate and minimize interferences. Hochman et al.[62] suggest that large scale screening for P-gp interactions may be an inefficient use of resources and might create perceived issues that have no significance in vivo. They suggest that it might be sufficient to limit assay use to characterizing lead candidates only.

Some compare flux in the overexpressing cell line versus the parent or wild-type cell line where increased asymmetry in the overexpressing cell line is interpreted as recognition by the expressed human P-gp. Occasionally, the lower level of endogenous canine P-gp expressed in the parent cell line is sufficient for some compounds to result in efficient polarized transport, and this can complicate interpretation. Others have used Caco-2 cells since this is a more commonly used cell model system for screening permeability characteristics.[76] Polarized efflux in this cell is often attributed to P-gp, but the level of P-gp expression in Caco-2 subclones used within each lab differs widely. Furthermore, many other uptake and efflux transporters are expressed by Caco-2 cells that could lead to confusing results, and this should be considered when using this approach to drive structural changes with the intent of circumventing P-gp.[77] For example, transcellular flux of the zwitterion sulfasalazine is highly polarized in Caco-2 cell monolayers, but this is attributed to basal membrane uptake by an unidentified organic acid transporter and apical efflux by an undefined transporter that does not appear to be P-gp.[78,79] In comparison, sulfasalazine is not polarized across MDCK cell monolayers because these apparently lack the rate-limiting organic acid transporter required for net transport. Polli et al.[66] provide rational criteria for selecting a P-gp substrate assay and interpreting the results by categorizing test compounds. They combined three different assays (bidirectional transport, ATPase activity, and the CAM uptake assay) to test 66 compounds. Category I compounds showed concordance between the three assays and were identified as P-gp substrates and inhibitors. Loperamide, terfenadine, and quinidine are representative positive category I control compounds for all three assays. Amantidine and triampterin are recommended as negative controls. Category II compounds showed assay differences based upon passive diffusion. For category IIA, fast passive diffusion resulted in a false negative, e.g., absence of polarity, in the bidirectional substrate assay. The ATPase and CAM assays provided more reliable data. For category IIB, slow passive diffusion resulted in a false negative in the ATPase and CAM assays and the bidirectional substrate assay performed more reliably. A similar categorization was proposed by Raub et al.[80] using a substrate assay and the CAM assay to identify when inhibition might contribute to a false negative. In this instance, the categories are defined on the basis of the passive permeability of the test compound and the magnitude of its apparent buffer/cell distribution coefficient.

The efficiency of the efflux pump is a product of the rate of passive diffusion, the residence time for that compound at the pump, i.e., within the membrane environment, and the strength of the molecular interaction between compound and pump, e.g., K_m. One begins to see then how cell (membrane) partitioning can complicate measuring a true K_m since local concentrations at the pump can be very high relative to extracellular or even total cellular concentrations. Well-partitioned compounds with high K_m values (low affinity) may be efficiently pumped. The consequences of this are 2-fold with regard to practically driving a chemistry strategy for circumvention of P-gp. Instead of altering the K_m to reduce P-gp efflux, one can either increase passive diffusion or reduce cell (membrane) partitioning. Additional scaffold-dependent attributes such as poor aqueous solubility and loss to plastic surfaces also have profound effects.

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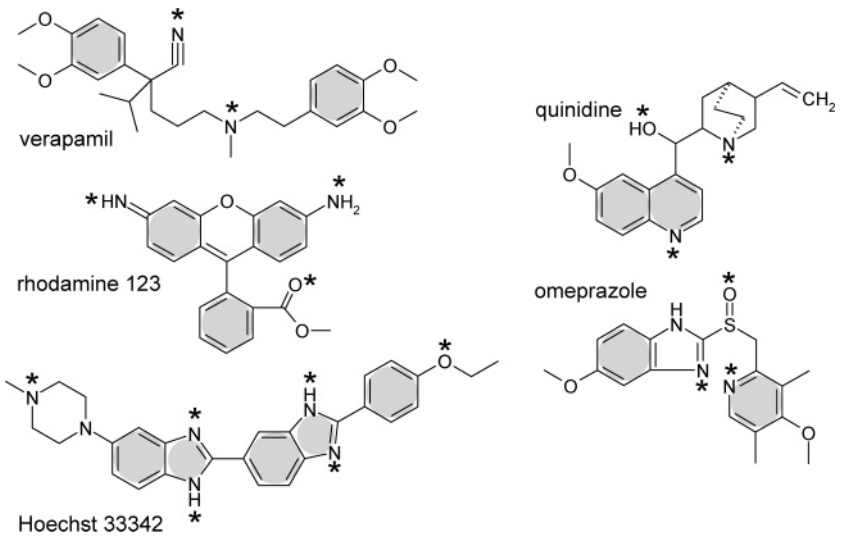

Figure 3. Example P-glycoprotein substrates with pharmacophore contact points identified according to Pajeva and Wiese.[84] Shaded areas are hydrophobic contact points, and predicted H-bonding groups are marked (*).

4. Structure−Activity Relationships

Chemoinformatics and Predictive Models. There have been a number of studies over recent years that define generic chemical features that are in common to compounds that interact with P-gp. It is not the intent here to provide a critical review of these studies. Stouch and Gudmundsson[10] provide a nice critique of what is known about P-gp and structure−activity relationships and list the 23 data sets up to that point in time that had been used in such studies. They also point out the caveats associated with these data sets, such as the emphasis on P-gp inhibition, not substrate activity, lack of structural diversity, assay complexities, and data inconsistencies. Nevertheless, we can easily paint a general picture of what a P-gp substrate looks like: it is lipophilic, not just hydrophobic; it has a large size or molecular volume; it is amphiphilic with the occasional presence of a weakly cationic group; it contains electronegative groups contributing dipole moment and hydrogen bonding groups.

There are several molecular models claiming to be reasonably accurate at predicting P-gp substrates and/or inhibitors. The majority of these models are pharmacophore-defined from three-dimensional quantitative structure−activity relationships (3D-QSAR).[55,81−88] These models generically define the virtual binding site in broad terms, but limited scope, from relatively small numbers of compounds.[10] Differences occur, especially in the molecular distances between contact points, because of different assay methods, compound training sets, and software used. Unfortunately, rarely do the authors compare and contrast their model with preceding models. Most of these models identify the minimal requirements to involve one or two hydrophobic centers including one or two aromatic rings, one to three H-bond acceptors (>N−, −OH, ═O), and/or one H-bond donor (>N−, −OH, ═NH, −NH−). Pajeva and Wiese[84] further considered the strengths of the H-bond groups, rather than just their number, by calculating H-bond acceptor capacities. The concept is that binding affinity depends upon appropriate spatial arrangement of their hydrophobic and polar elements, rather than their chemical motifs, that meet the P-gp requirements for recognition.[55] The more active ligands should have more pharmacophore contact points simultaneously occupied and the more binding patterns observed[84] (Figure 3). For example, efficient ligands such as vinblastine,

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verapamil, and Rhodamine 123 have four to six functional groups, whereas weaker ligands such as quinidine and omeprazole have three to four functional groups involved in these pharmacophore contact points. Thus, it is not ligand size but the concomitant increase in the probability to find interactive groups that determines the P-gp binding. The observed involvement of different, but closely located, atoms belonging to conformationally flexible groups suggests that the pharmacophore points are flexible. Moreover, a putative, large binding site appears to undergo conformational changes so that both protein and drug adopt each other in the best way.[84] These conclusions are consistent with the concept of two asymmetric drug binding sites on P-gp and the widely diverse structures recognized by P-gp.[54,56] Accordingly, the results of Garrigues et al.[55] deserve mention because two different, but partially overlapping, pharmacophores were described. Pharmacophore one binds verapamil, cyclosporin A, and actinomycin D and contains one aromatic area, two alkyl areas, and one electron donor. Pharmacophore two binds vinblastine and contains one aromatic area, three alkyl areas, and one electron donor. These pharmacophores are juxtaposed and share some common contact sites where ligand size affects their ability to compete with other ligands, consistent with a singularly large region on P-gp that can bind more than one ligand at a time.

While some of the prediction accuracies for test sets have been remarkably good, there has not been an explosion in their use as judged by the absence of literature demonstrating their practical application in driving SAR for P-gp circumvention. This may be an indication of the absence of global applicability for extrapolating models into other chemistry space, but it has been stated that the information from such models is of low value in driving SAR.[10] However, all agree that rapid and efficient computational methods are needed because the in vitro assays are too costly and time- and resource-consuming. Thus, computational models can be used to prioritize and reduce the number of compounds tested in these labor-intensive screening assays.

Other studies involved correlations of P-gp substrate activity to molecular descriptors.[64,89−99] Many more models that focus on P-gp modulators, and are not listed above, have been described. Bain et al.[89] suggested that substrates tend to have an H-bond potential of >8. In the same study, antagonists had a dipole moment of ≥3.3 as measured by the DelRe method. Seelig[93,94] was more explicit in defining the number of electron donor groups and their fixed spatial distance as deduced from a database of 100 P-gp ligands. Electron donor groups (Figure 4) include electronegative atoms (O, N, S, F, or Cl) with an unshared electron pair and groups with a π-electron orbital of an unsaturated system. Two patterns of electron donor groups were defined. Type I units have two electron donor groups separated by a

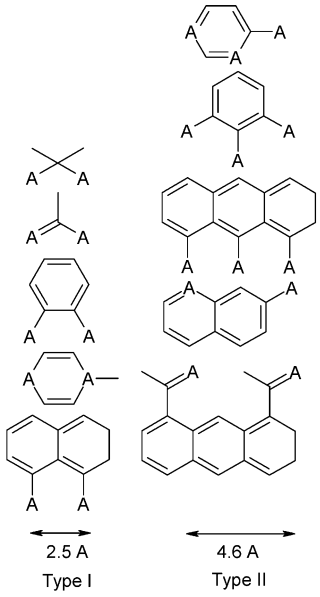

Figure 4. Proposed P-glycoprotein recognition of electron donor group (A) patterns and their fixed spatial distance.[93,94] Type I units have two electron donor groups separated by a distance of 2.5 ± 0.3 Å. Type II units have two or three electron donor groups where two groups are spaced 4.6 ± 0.6 Å apart.

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distance of 2.5 ± 0.3 Å. Type II units have two or three electron donor groups where two groups are spaced 4.6 ± 0.6 Å apart. Seelig[93,94] proposed that ligands contain at least one type I or type II unit, but may contain both. She also suggested that compounds with at least one type II unit can induce expression of P-gp. Electron donors were any groups with an unshared electron pair on an electronegative atom, e.g., O, N, S, F, or Cl. The following groups were identified as contributing to P-gp interactions from most to least frequently observed in P-gp ligands: >C═O, −O−, $-NR_2$, −NHR, −OH, −N═, R−halide, −S−, $-NH_2$, $>C(Phe)_2$. Of these, the alkoxy moiety, in combination with another carboxyl or carbonyl (to form an ester), appeared most often as a component of the proposed type I and II units. The hydroxy moiety occurred less frequently and typically in association with a carbonyl or a tertiary amine. Ecker et al.[100] followed up on Seelig's[93,94] findings and suggested a correlation between the summed electron donating strength of a ligand and its potency as an inhibitor. Other contributing factors were likely canceled out by examining a congener series.

Again, such information is generally valuable providing guidelines to structural modifications, but is of limited use for designing analogues a priori to methodically drive SAR away from P-gp limits.[10] A more recent, novel approach differing from these classical pharmacophore models was described by Gombar et al.[101] They used electrotopological state (ES) values in a unique way, the "Gombar−Polli molecular E-state (MolES) P-gp rule", that quantifies the impact of each atom in the context of near neighbors such that this approach may have more potential for designing analogues. An 86% prediction accuracy is claimed for binary discrimination of 58 compounds as substrates and nonsubstrates, where compounds with a MolES value of >110 were mostly substrates and those with a MolES value of <49 were nonsubstrates. Five mispredictions were explained by assay limitations such as fast passive diffusion and P-gp inhibition at the concentration tested for transport. It is likely that the indeterminate zone between MolES values of 50−110 will be redefined as other scaffolds representing unique chemical space are examined. It has been suggested here and elsewhere that future computational models should consider passive permeability in parallel to the active transport.[62]

Likewise, it also is clear that the lipid phase of the membrane cannot be overlooked while investigating P-gp activity[61,64] (section 2). Most computational models include the membrane interacting attribute of P-gp ligands by default, but some suggest it too should be modeled separately from transport and passive diffusion.[10] The binding of substrate to P-gp also might be modulated by properties of the lipid membrane.[102] Substrate−P-gp interaction is dependent upon the dielectric constant of the local environment.[49] van der Waals interactions are less pronounced within the lipid versus aqueous environments, and electrostatic or dipolar interactions are thought to be increased due to the low dielectric constant of the membrane lipid core region. Weak electrostatic interactions, e.g., between π-electrons of aromatic rings and a cation or H-bond interaction, that can be considered as dipole−dipole interactions may become more important within the membrane environment.[49] Thus, correlated descriptors should be considered from the perspective of their activity within a solvent akin to the membrane.

P-Glycoprotein Circumvention Using Rational Design. The task of rationally designing a chemistry strategy for circumventing a limiting P-gp interaction can be intimidating and frustrating. First, the necessity of retaining biological potency and metabolic stability places restrictions on what can be done. Second, the SAR for P-gp is obviously complicated and poorly understood, which prevents using a single, measurable value to monitor effective SAR changes. This review will use examples illustrating how certain modifications to a variety of chemical scaffolds can change the efficiency of the P-gp efflux pump contribution. Less attention is given to the way in which these changes were assessed, or what assays were used to measure the change in P-gp efficiency. This is an important point because the outcome is specifically defined as "efficiency" and may or may not involve an effect on the ligand−protein interaction per se.

The parameters within the assay that affect pump efficiency, such as passive diffusion, membrane partitioning, and molecular interaction between pump and substrate, should be understood when interpreting data sets associated with chemistry around a scaffold. Pump efficiency decreases as passive diffusion increases. Pump efficiency also can decrease when passive diffusion becomes so slow that access of compound to pump is rate limiting. Membrane partitioning can have a positive effect on P-gp-mediated transport such that local concentration at the pump is increased. Experience tells us that it is best to begin by surveying the extremes of chemistry space within the scaffold so that boundaries of behavior are identified. One should not limit the compounds tested to only those with potent activity, so that a chemistry space bias is not imposed. Once the boundaries are identified, testing of analogues should be purposeful with distinct moiety comparisons to probe microdomains of the scaffold. Examples are demonstrated for a scaffold represented in Figure 5.[80] Substitutions around X and Y either had no effect or had some impact on buffer/cell partitioning, but P-gp efflux was unaffected. Secondary substitutions on X, especially those with H-bonding capacity to increase activity, tended to decrease passive diffusion and buffer/cell partitioning, but did not alter net P-gp efflux. Single deletions, as shown here

(100) Ecker, G.; Huber, M.; Schmid, D.; Chiba, P. The importance of a nitrogen atom in modulators of multidrug resistance. *Mol. Pharmacol.* **1999**, *56*, 791−796.

(101) Gombar, V. K.; Polli, J. W.; Humphreys, J. E.; Wring, S. A.; Serabjit-Singh, C. S. Predicting P-glycoprotein substrates by a quantitative structure−activity relationship model. *J. Pharm. Sci.* **2004**, *93*, 957−968.

(102) Romsicki, Y.; Sharom, F. J. The membrane lipid environment modulates drug interactions with the P-glycoprotein multidrug transporter. *Biochemistry* **1999**, *38*, 6887−6896.

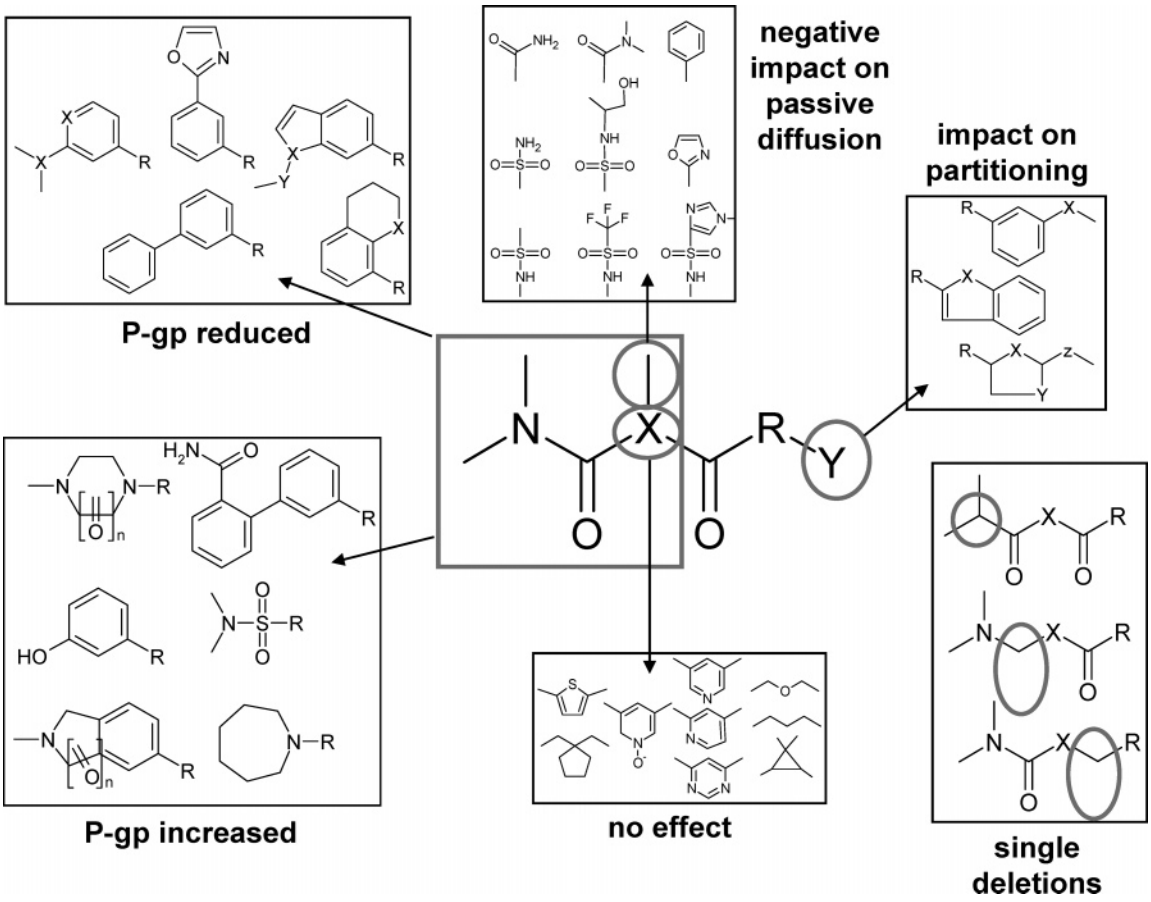


Figure 5. Assessment of substitutions and deletions in a peptidomimetic scaffold to deconvolute attributes that increase/decrease P-glycoprotein transport efficiency. Bidirectional flux across MDCK-MDR1 cell monolayers was measured for over 100 analogues.

for nitrogens and carbonyls, can be helpful to pinpoint dominating positive effects on P-gp efficiency. In this case, the results implicated a need to pursue different heterocycles with fewer H-bonding groups to lower P-gp-mediated efflux. It also was evident that P-gp pumping efficiency in untreated control MDCK-MDR1 cells was decreased (AB direction apparent permeability coefficient (P_{app}) increased) as passive diffusion (MDCK-MDR1 cells pretreated with P-gp inhibitor) increased (Figure 6). The compounds in the upper, right quadrant of this plot had the desirable characteristics to better overcome limiting P-gp efflux, and these generally had a 30% reduction in molecular size, fewer H-bonding groups, and reduced cell partitioning.

As one applies the in vitro assays, it is imperative that the results be benchmarked to in vivo performance.[30,103] In the example shown in Figures 5 and 6, the rate of brain uptake was limited by P-gp-mediated efflux.[80] This was confirmed by demonstrating rapid brain uptake in mdr1a(−/−) mice that are deficient in P-gp. A portion of the compounds that showed lessened P-gp-mediated efflux in vitro were tested

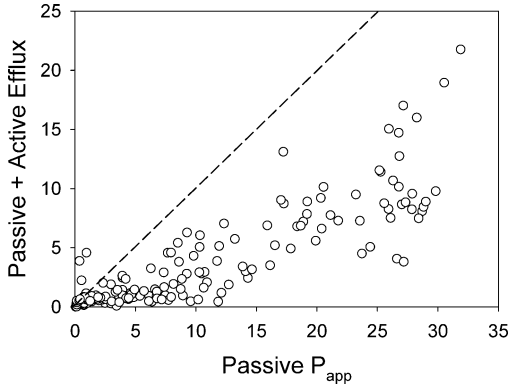


Figure 6. Mean apparent permeability coefficients ($P_{app} \times 10^{-6}$ cm/s) for 194 structurally related peptidomimetics. These were measured in the apical-to-basal direction across (i) MDCK-MDR1 cell monolayers pretreated with an equipotent analogue of P-glycoprotein inhibitor LY335979 at 2.5 μM (x axis) to give intrinsic membrane passive diffusion and (ii) untreated MDCK-MDR1 cell monolayers (y axis) to give passive diffusion plus P-glycoprotein-mediated efflux. The dashed line is the hypothetical slope of 1 emphasizing that this series is dominated by active transport.

in vivo with the expectation that the moiety changes would result in an increase in rate of brain uptake (Figure 7). This was done using a calibrated mouse brain uptake assay

(103) Yamazaki, M.; Neway, W. E.; Ohe, T.; Chen, I.; Rowe, J. F.; Hochman, J. H.; Chiba, M.; Lin, J. H. In vitro substrate identification studies for p-glycoprotein-mediated transport: species difference and predictability of in vivo results. *J. Pharmacol. Exp. Ther*. **2001**, *296*, 723−735.

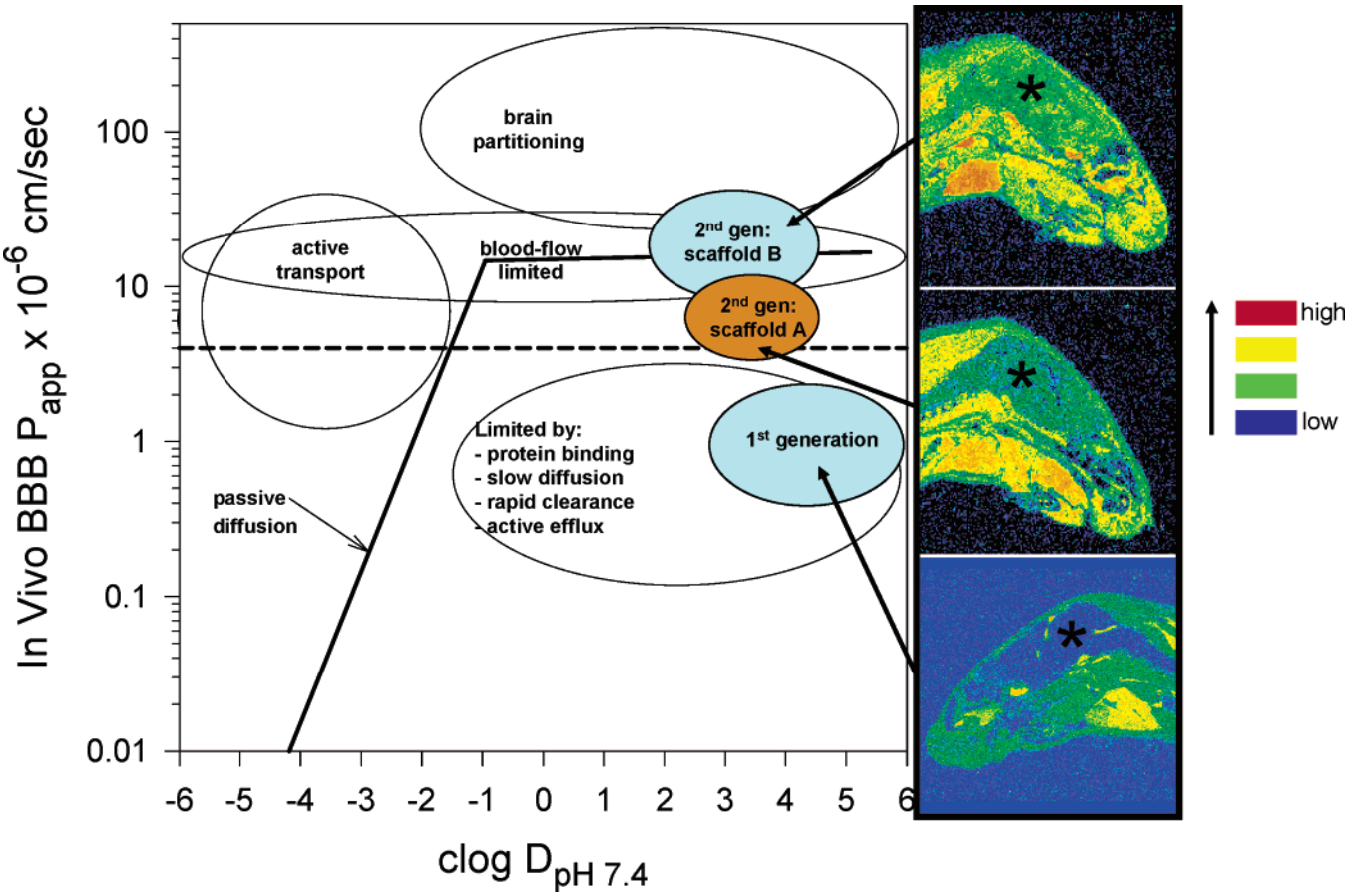


Figure 7. The example in Figure 5 enabled a redirection of chemistry from first to second generation (two scaffolds) compounds with improved brain uptake. Brain distribution volume and blood−brain barrier (BBB) permeability were increased. This is illustrated in the digitized images of autoradiograms from heads of mice that were dosed intravenously 30 min before with three different, tritiated analogues representing each scaffold. The progressive increase (scale shown) in brain-localized radioactivity (*) is associated with the increase in BBB apparent permeability coefficient (P_{app}) estimated from a brain/plasma ratio 5 min after an intravenous dose. The identified categorical regions of mechanistic interpretation were determined from the behavior of assorted calibrating solutes as described in the text. A log *D* was calculated using PALLAS version 3 software.

(MBUA) that is a modified two-compartment, single-dose intravenous administration method for assessing BBB permeability. A permeability−surface area coefficient (PS, $cm^3 \cdot g^{-1} \cdot s^{-1}$) was estimated from a single, 5-min time point and an apparent in vivo blood-to-brain or BBB permeability coefficient (P_{app}) calculated assuming a surface area of 240 cm^2/g of brain tissue. Only parent compound was analyzed in triplicate mice with mass spectrometry detection. The method was calibrated against solutes reported using other methods in rats,[80] and these defined the categorical regions of mechanistic interpretation shown in Figure 7. Compounds that diffuse across the BBB passively are shown by the solid line. This behavior reaches a maximum where the brain/plasma ratio is ∼1 because the rate of efflux equals the rate of influx. This region is limited only by the cerebral blood-flow rate. Compounds that exceed this maximum P_{app} are binding or partitioning into the brain tissue, or rarely for small organic molecules, within the capillaries themselves. Compounds that are too hydrophilic to diffuse passively and rapidly across the BBB can be actively transported via many different kinds of transport systems. Compounds that are hydrophobic enough to diffuse passively and rapidly across the BBB are often limited by one or a combination of other factors such as serum protein binding, poor diffusion within the parenchyma, rapid systemic clearance, or active efflux. In this example case, we targeted a BBB P_{app} value of ∼4 × 10^{-6} cm/s, or a brain/plasma ratio of ∼0.3 (Figure 7). The range of this series had been profiled by testing compounds that were inactive, but were likely to define the boundaries for behavior of this scaffold. Using the MBUA along with 3-in-1 cassette dosing,[80] iterative testing aided chemistry toward several, second-generation scaffolds that showed marked improvement in brain uptake (Figure 7). The increased permeability was confirmed to coincide with an increase in distribution volume using concomitant imaging. Selected analogues representing the scaffolds were tritiated and dosed in mice, and total radioactivity was imaged using whole-body autoradiography. HPLC radiodetection also was done to confirm that brain-associated radioactivity was mostly parent compound. Distribution of compound throughout the parenchyma was important for establishing that increased permeability assured access to the target. In this

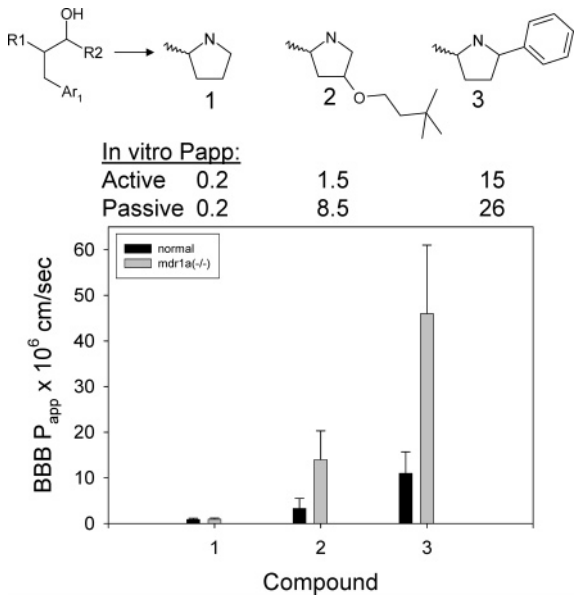


Figure 8. Three structurally related (R_1 and Ar_1 are identical) pyrrolidines (R_2 = **1**−**3**) were tested in vitro and in vivo in the presence and absence of P-glycoprotein transport. The mean apparent permeability coefficients ($P_{app} \times 10^{-6}$ cm/s) were measured in the apical-to-basal direction across MDCK-MDR1 cell monolayers without (active) and with a selective inhibitor of P-glycoprotein (passive). The blood−brain barrier (BBB) P_{app} (mean and standard deviation; $N = 3$) was measured as described in Figure 7 using normal control and P-gp-deficient (mdr1a−/−) mice.

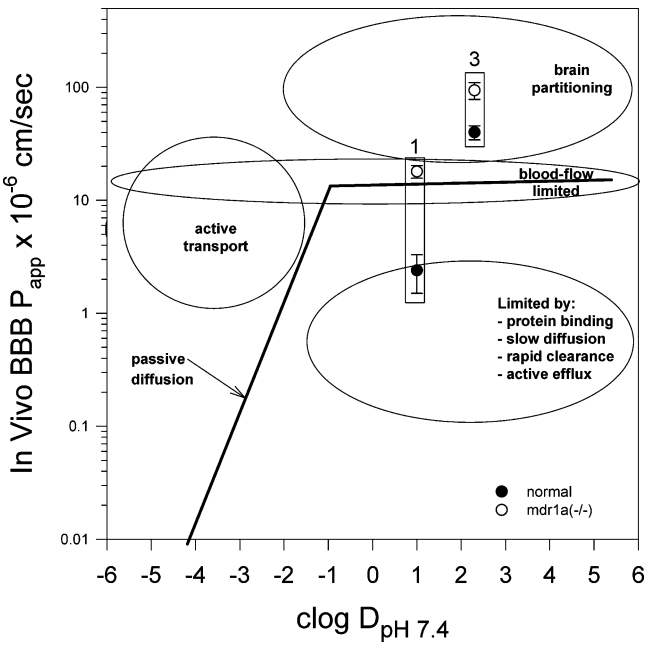


Figure 9. Performance of biarylpyrrolidine analogues **1** (secondary amine) and **3** (tertiary amine) in vivo. The blood−brain barrier (BBB) apparent permeability coefficients (P_{app}; mean and standard deviation; $N = 3$) was measured as described in Figure 7 using normal control and P-gp-deficient (mdr1a−/−) mice.

case, the target was intracellular rather than at the cell surface, which required that the compound have a large distribution volume within the brain. This exemplifies the value of applying imaging methods earlier in the discovery process.

Figure 8 demonstrates the impact of increasing passive diffusion of three pyrrolidine congeners.[104] Compound **1** was too hydrophilic to cross a membrane rapidly, and this is reflected in the very slow passive permeability in vitro and in vivo when P-gp is absent. In this case, access to P-gp most likely is rate-limited, resulting in the absence of net efflux. Substitution of the pyrrolidine with an alkyl ether (compound **2**) increased passive permeability (0.2 to 8.5 $\times$ 10^{-6} cm/s) and manifests a P-gp effect as demonstrated by the difference in brain uptake between normal control and mdr1a(−/−) mice. The increased hydrophobicity enhances membrane partitioning and access to pump, but the ether could add a P-gp recognition site to this template. Phenylation of the pyrrolidine (compound **3**) nicely shows that the marked increase in passive permeability (26 $\times$ 10^{-6} cm/s) in vitro decreased the efficiency of P-gp-mediated efflux (15 $\times$ 10^{-6} cm/s). This was effective at increasing the rate of brain uptake despite that compound **3** is a P-gp substrate as shown by the increased brain uptake in mdr1a(−/−) mice.

In another example, six congeners were carefully selected from a series of H-bond substituted biarylpyrrolidines to cover an anticipated array of functional groups that might be expected to be involved in P-gp interactions (see Chemoinformatics and Predictive Models subsection above). These were tested in the P-gp substrate assay composed of MDCK-MDR1 cells without and with a selective and potent P-gp inhibitor.[105] The results showed that the presence of a secondary amine (compound **1**) was sufficient for P-gp recognition as a substrate (Figures 9 and 10). Tertiary amine containing analogues (compound **2**) were not transported by P-gp (Figure 10). Thus, N-alkylation of the secondary amine in compound **1** (compound **3**) resulted in a loss of net transport (Figure 10) and an improvement in brain exposure (Figure 9). The performances of these two homologues were compared in mdr1a(−/−) mice deficient in P-gp at the BBB confirming that P-gp-mediated efflux of compound **1** was more impacted than that of compound **3** (Figure 9). However, the slight increase in brain exposure for compound **3** implies that this analogue is still recognized as a substrate by P-gp, which affects its distribution volume within the brain. This emphasizes the underlying concept that no single functional group alone is recognized, but one group can accentuate the recognition points existing within a scaffold. It is likened to a rheostat, rather than an on/off switch, where addition or removal of a key group can increase or decrease the pumping efficiency. The secondary amine was sufficient, but not required, because analogues containing other H-bonding groups such as one carbonyl (compound **4**) were less affected in P-gp efflux by N-alkylation (Figure 10). In effect, P-gp

(104) Raub, T. J.; Sawada, G. A.; Staton, B. A. Unpublished.

(105) Raub, T. J.; Sawada, G. A.; Staton, B. A.; Mitch, C. Unpublished.

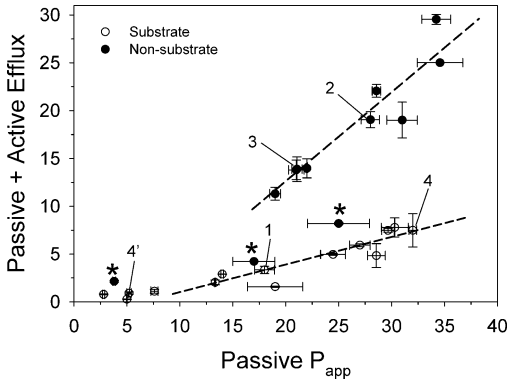


Figure 10. Mean (and range; $N = 2$) apparent permeability coefficients ($P_{app} \times 10^{-6}$ cm/s) for 27 structurally related biarylpyrrolidine analogues. These were measured as described in Figure 6. Compound **4′** is the des-*N*-alkyl derivative of compound **4**. P-glycoprotein (P-gp) substrates were distinguished from nonsubstrates by a flux ratio of ≥3. The three misclassified nonsubstrates (*) were caused by very high cell partitioning or P-gp inhibition.

recognition switched from the secondary amine to the carbonyl. Although N-alkylation had less impact on P-gp recognition in cases where another H-bonding group existed, it did in certain cases result in a marked increase in passive diffusion. This was shown by the increase in permeability for compound **4** compared to its des-*N*-alkyl homologue or compound **4′** (Figure 10). Armed with this information about these six tested congeners, a second group of analogues was selected to confirm the SAR conclusions and to explore options of tolerance. Of course, it helps when such iterative changes result in an improvement in efficacy (greater response at same dose or equal efficacy at lower dose) in vivo. As shown for the case study in Figure 6, here too an increase in passive diffusion, without an apparent decrease in P-gp recognition, resulted in decreased P-gp efficiency as shown by the increase in AB P_{app} in untreated control MDCK-MDR1 cells (Figure 10).[105]

A review by Ambudkar et al.[21] is essentially the only attempt to collect a group of studies for teaching SAR requirements of P-gp for a variety of chemical scaffolds. They primarily focused on P-gp inhibitors and optimization of their reversal effect. The chemistry included analogues of verapamil, reserpine, staurosporine, propafenone, phenoxazine, quinacrine, chloroquine, phenothiazines, and prenylcysteines. The most detailed study used colchicine analogues. Colchicine is a tricyclic scaffold of six- and seven-membered rings[106] (Figure 11). Methoxylation of the A- and C-rings at C-1, C-2, C-3, and/or C-10 had no effect on P-gp recognition. Replacement of the seven-membered C-ring with a six-membered ring decreased efficient P-gp recognition, as did elimination of the B-ring. Substitutions at position

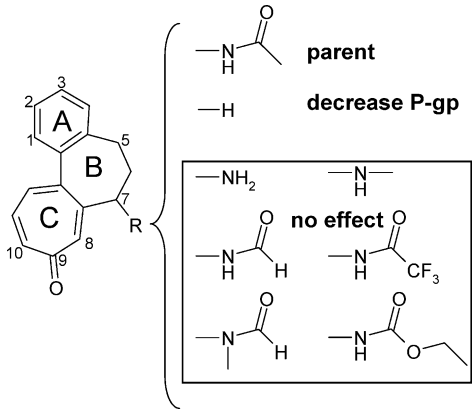


Figure 11. Example substitutions on colchicine and their effect on P-glycoprotein transport.[106]

C-7 of the B-ring, especially removal of the nitrogen atom, resulted in loss of P-gp recognition. Chain length, changing from an amide to a secondary amine, or addition of more electronegative fluorine groups involving the acetyl had minimal effect. Replacement of the acetamido with an amino group was equivocal, thus the N atom and not the acetyl group plays a key role. It was suggested that the nitrogen atom participates in π-aromatic electron interactions with the C-ring or the C-7-NH functions as an H-bond donor.

The importance of a charged basic amino group has been addressed in several other chemical scaffolds. Solary et al.[107] first showed that substitution of the C3′ amine group in doxorubicin with a hydroxyl group overcame P-gp-mediated resistance as indicated by a 20-fold increase in cytotoxicity (Table 1). Similarly, adriamycin analogues with the positively charged $-NH_2^+$ group replaced with $-OH$, or the $-NH^+$ replaced by an ether ($-O-$), were more effective in drug resistant cell lines; however, it was unclear if this was directly attributed to the removal of the positive charge or to an increase in hydrophobicity.[108] A decline in the importance of charge was associated with an increase in hydrophobicity. These studies could not determine whether these uncharged species were no longer recognized by P-gp, or rendered less efficient by other contributing factors. Additional studies with anthracycline analogues showed that the nitrogen atom does not interact with P-gp in the charged form, but rather acts as an electron donating group.[109,110] Introduction of a nitro group in the benzyl ring of daunomycin resulted in poor P-gp

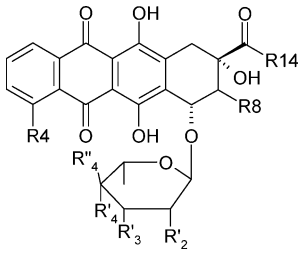
(106) Tang-Wai, D. F.; Brossi, A.; Arnold, L. D.; Gros, P. The nitrogen of the acetamido group of colchicine modulates P-glycoprotein-mediated multidrug resistance. *Biochemistry* **1993**, *32*, 6470−6476.

(107) Solary, E.; Ling, Y. H.; Perez-Soler, R.; Priebe, W.; Pommier, Y. Hydroxyrubicin, a deaminated derivative of doxorubicin, inhibits mammalian DNA topoisomerase II and partially circumvents multidrug resistance. *Int. J. Cancer* **1994**, *58*, 85−94.

(108) Lampidis, T. J.; Castello, C.; del Giglio, A.; Pressman, B. C.; Viallet, P.; Trevorrow, K. W.; Valet, G. K.; Tapiero, H.; Savaraj, N. Relevance of the chemical charge of rhodamine dyes to multiple drug resistance. *Biochem. Pharmacol*. **1989**, *38*, 4267−4271.

(109) Frezard, F.; Pereira-Maia, E.; Quidu, P.; Priebe, W.; Garnier-Suillerot, A. P-glycoprotein preferentially effluxes anthracyclines containing free basic versus charged amine. *Eur. J. Biochem*. **2001**, *268*, 1561−1567.

Table 1. Kinetic Parameters for a Series of Anthracycline Derivatives Measured as Cytotoxicity in Sensitive K562 and Resistant K562/Adr Cells[a]



anthracycline derivative	R_4	R_8	R_{14}	R'_2	R'_3	R'_4	R''_4	k_{out}[b]	k_{in}[b]
doxorubicin	OCH_3	H	OH	H	NH_2	OH	H	0.3	0.01
WP 697	OCH_3	H	OH	H	NH_2	H	OH	1.9	0.04
daunorubicin	OCH_3	H	H	H	NH_2	OH	H	1.5	0.2
WP 608	OCH_3	H	H	H	OH	NH_2	H	1.9	1.1
WP 656	OCH_3	H	H	H	NH_2	H	H	2.6	1.5
F-idarubicin	H	F	H	H	NH_2	OH	H	2.0	2.0
pirarubicin	OCH_3	H	OH	H	NH_2	pyran	H	6.2	3.5
idarubicin	H	H	H	H	NH_2	OH	H	1.9	4.0
WP 742	OCH_3	H	H	H	$N(CH_3)_2$	OH	H	3.4	4.0
WP 401	OCH_3	H	H	Br	NH_2	H	OH	4.7	7.0
WP 715	OCH_3	H	H	H	NH_2	F	H	1.3	13

[a] Marbeuf-Gueye et al.[69] [2] k_{out}, active efflux coefficient; k_{in}, passive influx coefficient (units are $\times 10^{12}$ L cell^{-1} s^{-1}).

substrate recognition, despite the presence of the protonatable secondary amine, whereas replacement of the secondary amine with a free amino group was well recognized despite not being protonated. Thus, although cationic groups are common in P-gp ligands, they are not required.

Both passive diffusion and membrane interactions must be monitored when circumventing P-gp. These key points were nicely illustrated by Marbeuf-Gueye et al.[69] where the passive influx coefficient, active efflux coefficient, and relative efficacy in resistant (K562/ADR) and sensitive (K562) cell lines were measured for 11 anthracycline derivatives (Table 1). All of these analogues contained an amine group, but varied with regard to H-bonding groups. A reduction in the number of −OH and $-OCH_3$ groups corresponded to the 1300-fold increase in passive influx, but these changes occurred in multiple sites that are unequal in their contribution (Table 1). The analogues with the slowest passive influx, doxorubicin and WP 697, were the most efficiently transported, and these analogues contained two −OH groups and one methoxy group. Doxorubicin had the slowest flip-flop rate and the lowest lipid phase/aqueous medium coefficient compared to daunorubicin followed by idarubicin. The position of the amine in WP 608 versus daunorubicin resulted in a 5-fold increase in influx, but the P-gp recognition appears to remain the same. The same seems true for loss of a methoxy group in daunorubicin to give idarubicin resulting in a 20-fold increase in influx, but little change in efflux. Using other approaches, idarubicin had 10-fold faster passive diffusion or a faster flip-flop rate, and a greater lipid phase/aqueous medium coefficient, compared to daunorubicin.[111,112] This is likely the reason Roovers et al.[113] claimed that idarubicin circumvents P-gp relative to other anthracyclines, although they attributed it to a greater lipophilicity. Halogenation is commonly used to increase membrane partitioning and permeability.[63] It was shown that substituting −H with −Cl and $-CF_3$ in two congener sets increases permeability 2−9-fold in artificial lipid membrane bilayers by enhancing the free energy of partitioning into the lipid membrane. Substitution of a −H group in WP 656 with fluorine to give WP 715 increased influx 8.7-fold; however, such was not the case with idarubicin and F-idarubicin involving a different position[69] (Table 1).

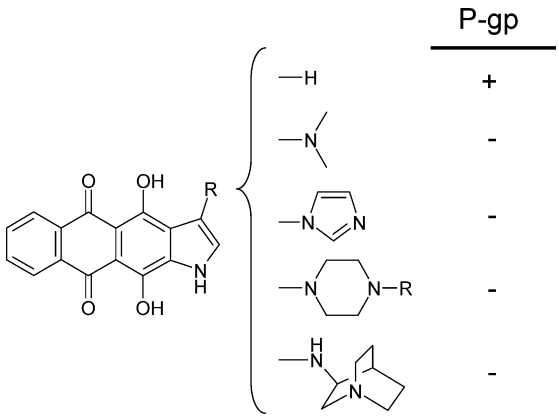


Figure 12. Example substitutions on 3-aminomethyl derivatives of 4,11-dihydronaphtho[2,3-*f*]indole-5,10-diones and their effect on P-glycoprotein transport.[114]

In an anthracycline-like series of 4,11-dihydronaphtho-[2,3-*f*]indole-5,10-dione, 3-aminomethyl derivatives, especially cyclic diamines such as unsubstituted and N-substituted piperazines, were equally active in parent and P-gp overexpressing, resistant K562 cell lines[114] (Figure 12). The mechanism for this apparent circumvention was not determined. One explanation is that these analogues are improved inhibitors of P-gp, as demonstrated for a series of 1-aza-9-

(110) Salerno, M.; Przewloka, T.; Fokt, I.; Priebe, W.; Garnier-Suillerot, A. Preferential efflux by P-glycoprotein, but not MRPl, of compounds containing a free electron donor amine. *Biochem. Pharmacol.* **2002**, *63*, 1471−1479.

(111) Loetchutinat, C.; Saengkhae, C.; Marbeuf-Gueye, C.; Garnier-Suillerot, A. New insights into the P-glycoprotein-mediated effluxes of rhodamines. *Eur. J. Biochem.* **2003**, *270*, 476−485.

(112) Regev, R.; Yeheskely-Hayon, D.; Katzir, H.; Eytan, G. D. Transport of anthracyclines and mitoxantrone across membranes by a flip-flop mechanism. *Biochem. Pharmacol*. **2005**, *70*, 161−169.

(113) Roovers, D. J.; van Vliet, M.; Bloem, A. C.; Lokhorst, H. M. Idarubicin overcomes P-glycoprotein-related multidrug resistance: comparison with doxorubicin and daunorubicin in human multiple myeloma cell lines. *Leuk. Res.* **1999**, *23*, 539−548.

(114) Shchekotikhin, A. E.; Shtil, A. A.; Luzikov, Y. N.; Bobrysheva, T. V.; Buyanov, V. N.; Preobrazhenskaya, M. N. 3-Aminomethyl derivatives of 4,11-dihydroxynaphtho[2,3-f]indole-5,10-dione for circumvention of anticancer drug resistance. *Bioorg. Med. Chem.* **2005**, *13*, 2285−2291.

oxafluorene inhibitors of cyclin-dependent kinase (CDK).[115] Addition of phenyl and −O−ethyl alkoxy groups in two spatially separate positions increased efficacy in P-gp resistant cell lines. Because it was thought that modulation of multidrug resistance is an important feature of CDK inhibitors, especially if cotherapy with anticancer drugs is anticipated, these analogues were examined for their ability to affect Rhodamine 123 uptake by the resistant cells.[115] Indeed, the P-gp circumvention was the result of P-gp inhibition.

This concept of P-gp transport efficiency inversely related to passive membrane permeability was described for a congener series of simple cationic aromatic alkypyridinium and nonaromatic alkylguanidinium analogues.[116] Growth inhibition in parent and resistant cell lines correlated with hydrophobicity or alkyl chain length (C2−C6) for the substituted guanidinium, but P-gp effect was absent presumably because they are not substrates. It is likely that these compounds are very permeable so that P-gp may be inefficient in their transport. In contrast, the alkypyridiniums are P-gp substrates and transport efficiency correlates with an increase in hydrophobicity or length (>C4) of the alkyl chain. Similarly, Loetchutinat et al.[111] used seven rhodamine analogues to demonstrate that tetramethylrosamine with the least H-bonding groups was the best P-gp substrate despite having fast passive diffusion because of the increased P-gp recognition. The same conclusion was made by Eytan et al.[70] although experimental conditions differed.

Another example scaffold with significant literature about P-gp circumvention involves paclitaxel analogues or taxanes (reviewed by Ojima et al.[117] and Fang and Liang[118]). Almost all of these describe substitutions or modifications involving the C-10 position, which has no impact on intrinsic pharmacological activity. It is possible that the C-10 position is particularly important for P-gp interaction because there are many other groups within this molecule that can act as pharmacophore binding points (see Chemoinformatics and Predictive Models section). Ojima et al.[119] described a series

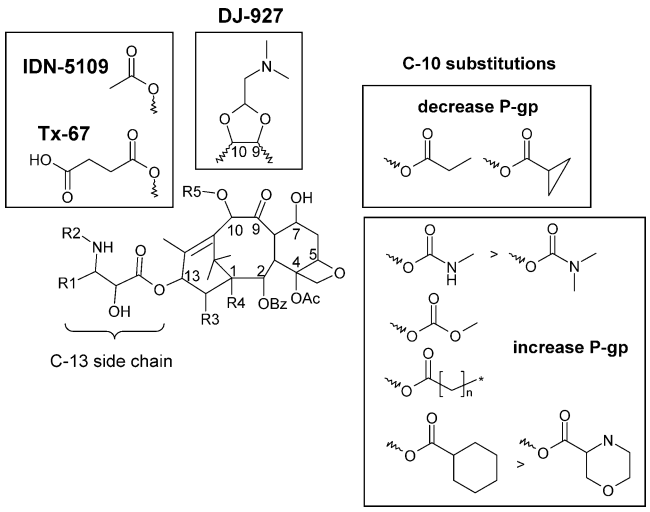


Figure 13. Example C-10 substitutions on taxane analogues and their effect on P-glycoprotein transport. The C-13 side chain is critical for tubulin binding activity.[117,118]

of 3′-(2-methyl-1-propenyl) and 3′-(2-methylpropyl) taxoids with 19 C-10 modifications where acetyl analogues are more active in resistant cell lines (Figure 13). Adding aliphatic or aromatic H-bonding groups and more hydrophobicity decreases this effect, or presumably increased P-gp recognition. A secondary amine versus a tertiary amine appeared less able to overcome P-gp. This SAR led to IDN-5109 that is in clinical trials as an orally active semisynthetic taxane analogue[117,120] (Figure 13). The carbonyl, consistent with its identification as one of the most common H-bonding groups in P-gp ligands, of the acetyl group was very important. However, like the anthracycline examples discussed above, the decreased effect of P-gp efflux on overcoming resistance is attributed to the improved ability of IDN-5109 with an acetyl group in position C-10 to inhibit P-gp, or to self-modulate.[121] A cyclized C-9/C-10 modification in DJ-927 also circumvented P-gp resistance, but the reason for this was not determined[122] (Figure 13). A more recent example, Tx-67, contains a succinyl substitution in the C-10 position, and data were collected directly demonstrating a reduction in P-gp transport in the absence of P-gp inhibition[123] (Figure

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(123) Rice, A.; Liu, Y.; Michaelis, M. L.; Himes, R. H.; Georg, G. I.; Audus, K. L. Chemical modification of paclitaxel (Taxol) reduces P-glycoprotein interactions and increases permeation across the blood-brain barrier in vitro and in situ. *J. Med. Chem*. **2005**, *48*, 832−838.

13). Tx-67 did not affect accumulation of Rhodamine 123 by cultured bovine brain capillary endothelial cells. Moreover, there was an 11-fold increase in transcellular permeability in vitro without concentration dependence and without effect by the P-gp inhibitor cyclosporin A. This combination of data is required to unequivocally confirm the reason for P-gp circumvention as reduction in substrate recognition. Further studies with Tx-67 were the first confirming that a C-10 substitution results in a 7−10-fold increase in BBB permeability in rat brain and improved efficacy in vivo versus paclitaxel.[124]

Analogues of antimicrotubule agent hemiasterlin, a tripeptide from marine sponge, were systematically examined for substitutions at different locations throughout the scaffold not only for intrinsic cytotoxicity but also activity (IC_{50}) in drug resistant KB cell lines.[125] The monocarboxylate HTI-286 circumvents P-gp resistance compared to paclitaxel, but as a peptidomimetic it is still a P-gp substrate rich in H-bonding groups. The results are difficult to interpret since intrinsic activity is changing greatly with some of the substitutions. Some observations are that a thienyl ester appeared to be a better P-gp substrate than the acid. A *m*-tolyl substitution of the amino-terminal A-segment had improved activity presumably due to P-gp circumvention; however, P-gp inhibition was not examined.[126] The *m*-tolyl could increase cell partitioning such that local concentrations are high. An indole in this position appeared to be better recognized compared to a phenyl.[126] The A-piece amine group was important for intrinsic activity, but there seemed to be no preference by P-gp for a secondary or tertiary amine. Last, loss of the CD-piece olefin, which is thought to confer rigidity, appeared to increase P-gp recognition, which may be indicative of a preferred conformation (see below).

A good example of P-gp circumvention attributed to a single structural modification is the study of two structurally related neurokinin-1 (NK-1) receptor antagonists, CP-122721 and CP-141938[30] (Figure 14). The latter analogue contains a terminal methylated sulfonamide that confers P-gp recognition. As a consequence, CP-141938 was less active in vivo despite a similar potency in vitro. This was shown to be due

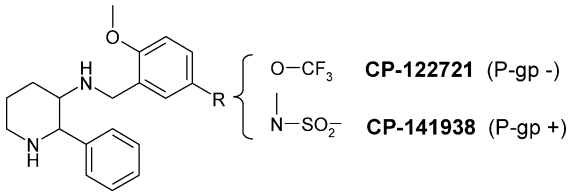

Figure 14. Structurally related neurokinin-1 (NK-1) receptor antagonists.[30]

to a 13-fold reduction in brain exposure. This study illustrates the importance of combining several in vitro assays of P-gp activity and in vivo data to confirm physiological relevance.[30] Both analogues stimulated P-gp ATPase with similar K_m values, but differences in their P-gp-mediated transport in two assays were dramatically different. This difference in efflux was replicated in vivo using the FVB mdr1a/b(−/−) mice deficient in P-gp, where brain exposure of CP-141938 was decreased ~40-fold by P-gp efflux. The experiment that distinguishes this from many other studies is the implication this has to pharmacodynamics where efficacy of CP-141938 required a 10-fold lower dose range after pretreating with a P-gp reversal agent to give an equivalent response to CP-122721. This study epitomizes how such studies should be conducted when assessing the importance of the chemistry changes made for circumventing P-gp.[30,127]

Camptothecin, topotecan, and irinotecan are topoisomerase I inhibitors for treating cancers, but are suspect to clinically relevant drug resistance. As such, non-camptothecin-like analogues of ARC-111 were explored for their improved activity in resistant cell lines attributed to P-gp and another ABC efflux transporter BCRP (ABCG2).[128] A series of *N*-alkyl-*N*-isopropyl analogues were found to not be substrates for P-gp relative to topotecan (Figure 15). The primary amine was not a substrate whereas the ARC-111 metabolite, an *N*-monomethyl secondary amine, was a P-gp substrate. Tertiary amines were not P-gp substrates including cyclized amines, except those introducing a second, basic amine such as 2-piperazinyl reinstated P-gp, but not BCRP.

Ueda et al.[20] summarized data for 10 steroids and implicated the importance of the C-11 hydroxyl group for P-gp transport. Thus, the des-OH homologue of P-gp substrate cortisol, or cortexolone, is not transported by P-gp. It appears that steroid P-gp substrates (cortisol, aldosterone, dexamethsaone, estriol) are more hydrophilic with the addition of more H-bond donating groups (−OH). Pardridge[129] showed that steroid uptake into brain was inversely

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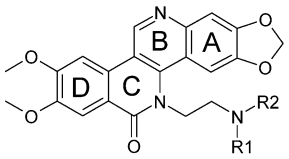


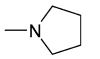
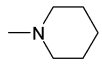
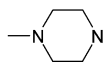
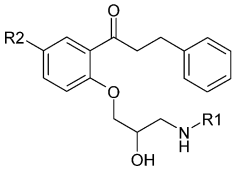
R1	R2	P-gp
H	H	NC
CH_3	H	+
CH_2CH_3	H	+
CH_3	CH_3	NC
H		NC
H		NC
H		+

Figure 15. Example substitutions on *N*-alkyl-*N*-isopropyl analogues of ARC-111 and their effect on P-glycoprotein transport.[127]

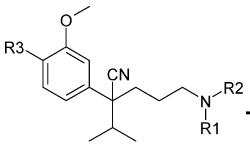
related to number of H-bonds (progesterone ∼ testosterone > corticosterone > aldosterone > cortisol). Thus, decreased passive diffusion and increased number of H-bond groups likely increased P-gp efflux and decreased BBB penetration. This was essentially confirmed by increased brain levels of cortisol > corticosterone > aldosterone > progesterone in P-gp-deficient mdr1a/b(−/−) mice.[130]

Other quasi-SAR studies involve comparisons of parent molecules and their metabolites. Risperidone appeared to be a better substrate for P-gp than its active metabolite 9-OH-resperidone on the basis of ATPase assay results.[131] Yet, brain exposure of both analogues appeared similarly increased as determined by disposition in P-gp deficient mice. Risperidone brain exposure in normal mice was markedly better than for 5-OH-resperidone, suggesting that 5-OH-resperidone has a more difficult time crossing the BBB, which might be attributed to slower passive diffusion.[33] While this called into question the accuracy of the ATPase assay for identifying P-gp substrates, it would not be surprising to see big differences between parent and metabolite attributed to small changes such as hydroxylation.[104] This effect is consistent with the synergy between efflux pumps and elimination of CYP enzyme products for detoxification.[40−42] Propafenone is metabolized to *N*-desalkylpropafenone by CYP3A4 and to 5-OH-propafenone by CYP2D6[132] (Figure 16). Parent and *N*-desalkylpropafenone do not appear to be P-gp substrates since polarized transport in Caco-2 cells is lacking, but 5-OH-



R1	R2		P-gp
CH_2CH_3	H	**parent**	-
H	H	**N-desalkyl**	-
CH_2CH_3	OH	**5-hydroxy**	+

Figure 16. Propafenone and its metabolites and their interaction with P-glycoprotein.[132]



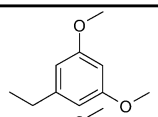
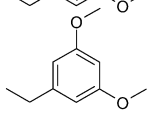
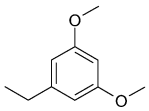
	R1	R2		P-gp
parent	CH_3		OMe	+
norverapamil	H		OMe	++
D-620	H	H	OMe	+++
D-617	H	CH_3	OMe	+++
D-703	CH_3		OH	+

Figure 17. Verapamil and its metabolites and their interaction with P-glycoprotein.[133]

propafenone is likely a P-gp substrate. In another example, Pauli-Magnus et al.[133] examined verapamil (Vpl) and four metabolites as substrates and inhibitors of P-gp (Figure 17). Vpl and D-703 were weak substrates and P-gp inhibitors, yet Vpl brain levels increased 20-fold in mice lacking P-gp. N-Demethylation of Vpl to norverapamil resulted in an increase in polarized transport and a decrease in IC_{50} for P-gp inhibition. Similar N-dealkylation of Vpl and norVpl, respectively, resulted in the terminal secondary amine D-617 and the terminal primary amine D-620. Both D-617 and D-620 had highly polarized transport in LLC-PK1 cells overexpressing MDR1, and neither was a potent P-gp inhibitor. Interestingly, the secondary amine D-617 was more polarized and had ∼5-fold slower passive diffusion than the primary amine D-620. All four metabolites were more hydrophilic than the parent Vpl, and passive diffusion was decreased by >2-fold in accord with addition of H-bonding groups. The impact of these changes on P-gp transporter efficiency is less clear in this case and may be further

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complicated by the ability of Vpl, norVpl, and D-703 to inhibit P-gp activity at relatively low concentrations, which can underestimate net efflux.[133]

Although there is no evidence for verapamil and norverapamil *R*,*S*-stereoisomers to display differences in P-gp transport efficiency,[134] there are studies with a few other compounds suggesting that P-gp substrate recognition can be stereoselective. Stereoselective inhibition of P-gp has been reported (see Hooiveld et al.[135]). Hooiveld et al.[135] demonstrated that the N-methylated monoquaternary salt of (*R*)-quinidine is a better substrate of P-gp than the N-methylated monoquaternary salt of (*S*)-quinine. It had already been shown that quinidine was a better inhibitor than its antipode quinine for P-gp, but these parent compounds were not used in this study.[136] The data also suggest possible species differences with human MDR1 transporting quinidine better than rat Mdr1b, but the K_m was not different, suggesting postbinding differences in the transporter proteins (80% amino acid homology).[135] These results show that quaternary salts can be pumped from the inner leaflet that is accessible in the inside-out vesicle system used here and consistent with the proposed shallowness of the binding sites in this membrane leaflet (section 2). In another example of stereoselectivity, brain exposures of the enantiomers of the antimalarial and neurotoxic drug mefloquine were compared.[137,138] The (−)-enantiomer had slower efflux from mouse brain, and pretreatment of mice with the P-gp inhibitor GF120918 demonstrated that the (+)-enantiomer was effluxed more efficiently from brain, consistent with it being a better P-gp substrate.[138] The (+)-enantiomer also is a better inhibitor of P-gp in vitro although species differences are suggested.[137] Prueksaritanont et al.[127] found a remarkable difference in P-gp transport of diastereomers of $\alpha_v\beta_3$-antagonists. The more hydrophobic diastereomer I (log P = 1.04) was a better P-gp substrate than the hydrophilic II (log $P = -0.05$) including apparently better recognition by mouse mdr1a P-gp versus human P-gp. Yet, this difference was not a factor in the difference in oral bioavailability between these diastereomers in rat. Gao et al.[139] showed that the *R*,*R*-configuration of hydroxyethylamine, used to stabilize against protease metabolism, containing peptidomimetics is preferred by P-gp. The dopamine receptor antagonist flupentixol shows stereoselectivity toward P-gp interactions.[140] The *cis*(*Z*)-isomer stimulates P-gp transport as an allosteric modulator, and the *trans*(*E*)-isomer inhibits P-gp.[141] Yet, both display equal binding potencies, and it was proposed that the stereoselectivity by P-gp is related to the different orientation of the molecules within the membrane lipid environment.[63] Siccardi et al.[142] reported that a series of aryloxy phosphoramidate triester prodrugs of the anti-HIV reverse transcriptase inhibitor 2′,3′-didehydro-2′,3′-deoxythymidine (d4T; stavudine) were P-gp substrates. The diastereoselective differences (SE > FE) seen in Caco-2 cells were concentration dependent and susceptible to the P-gp inhibitor verapamil, but surprisingly these differences were not observed in MDCK-MDR1 with increased expression of human P-gp. Stereoselectivity appeared to be absent when comparing daunorubicin and its antipode WP900,[143] and between peptidomimetic isomers of 1,2- and 1,3-aminodiols and azapeptides.[144] Thus, it remains to be proven that recognition of substrates by P-gp involves a high degree of stereoselectivity. It is likely compound dependent and might be confounded by the lipid environment displaying a high degree of structural discrimination through stereoselective interactions with phospholipids.[61] The present understanding of the flexible binding site(s) and its multiple pharmacophore contact points should diminish a general dependence on stereoselectivity for binding of substrates.

Another promising strategy to circumventing P-gp by altering the contact points associated with substrate−P-gp

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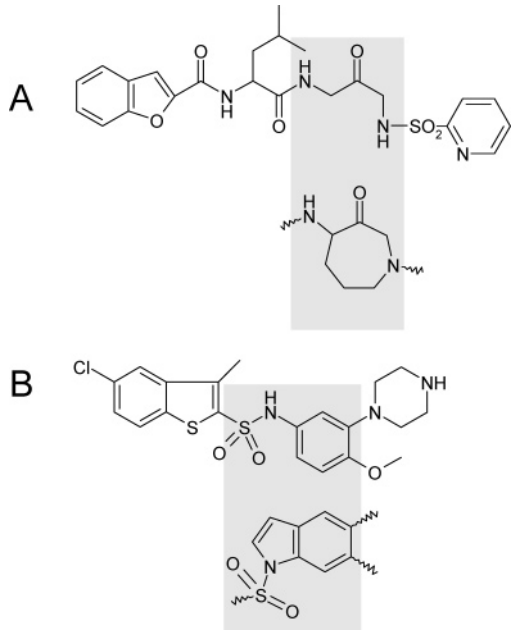

Figure 18. Examples of constrained conformation: (A) azepanone-based inhibitor of cathepsin K;[145] (B) bicyclic heteroarylpiperazine 5-HT_6 receptor antagonist.[146]

binding is conformational constraint. This is the introduction of a more rigid region in the molecule, and often concurrent with a reduction in H-bonding groups, that leads to an improvement in bioavailability. A few examples have emerged, and not all are linked directly to P-gp recognition. A series of azepanone-based inhibitors of cathepsin K were studied in Caco-2 cell monolayers and rat oral bioavailability.[145] The analogues had similar molecular weights, hydrophobicities, and H-bonding capacities. The acyclic derivatives were P-gp substrates, and conformational constraint via cyclization overcame this (Figure 18A). Introduction of a seven-membered ring and the loss of one H-bond donor resulted in an increase of oral bioavailability from 3% to 42% for the *S*-conformer. The *R*-isomer had only ~10% oral bioavailability, and this was thought to be attributed to a difference in metabolism. There was a concomitant decrease in polarized efflux too for both isomers versus the parent analogue. It was concluded that ring formation results in conformational constraint effectively locking the rotational freedom and limiting the number of conformations available to bind to P-gp. In another example, bicyclic heteroarylpiperazine 5-HT_6 receptor antagonists were conformationally constrained with the loss of one H-bond donor[146] (Figure 18B). A resulting 60-fold increase in brain exposure was attributed to increased plasma half-life and a reduction in P-gp transport measured using MDCK-MDR1 cells. Thus, conformational constraint appears to be a viable means to reduce P-gp recognition by lowering the number of conformations that are favorable for binding to the P-gp site, and with minimal loss of functional groups the promote activity.

Similar to conformational constraint is the intentional placement of intramolecular H-bonds. It has been proposed that loss of H-bonding groups through either removal or substitution (alkylation) of these groups can lower the energy required for desolvation and increase diffusion through a lipid membrane bilayer.[147] H-bonding groups can also be sterically shielded by introducing bulky groups adjacent to them, or by facilitating intramolecular H-bonds. Most examples for improved bioavailability do not involve P-gp efflux, but the benefits of the concept remain. A series of [2,3-*d*]pyrimidine-2,4-dione derivatives containing a biaryl moiety were studied as non-peptide human luteinizing hormone-releasing hormone (LHRH) receptor antagonists.[148] A urea substitution that improved potency significantly resulted in 10-fold less systemic exposure after oral dosing compared to the amide. The more stable cis-conformation of the methoxyurea (TAK-013) presumably improved permeability, because of the intramolecular H-bond between the aniline NH and the methoxy oxygen (Figure 19A). Unfortunately, there were no in vitro permeability assays done to corroborate this conclusion. Conradi et al.[149] described a series of pyridylcarboxamide regioisomers where the 2-pyridyl isomer was much more permeable than either the 3- or 4-pyridyl isomers. They used partitioning behavior between two solvent systems to demonstrate that in these membranelike solvents the 2-pyridyl congeners have greater partition coefficients, and this was attributed to either steric interactions or intramolecular H-bonds. Tryptophan-containing neurokinin-1 receptor antagonists were shown to have modest brain and poor aqueous solubility; thus, a second effort improved on these attributes.[150] Brain exposure was increased 10-fold by intro-

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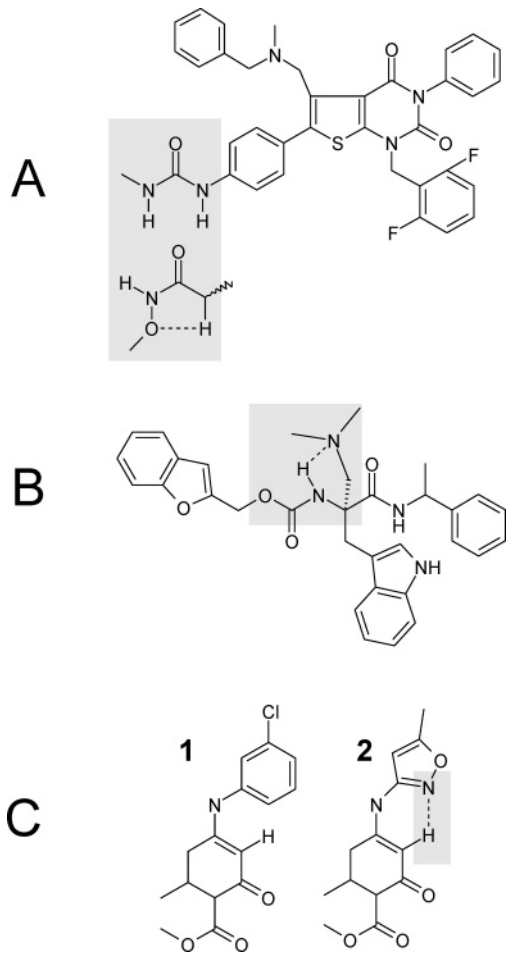

Figure 19. Examples of intramolecular hydrogen bonds: (A) [2,3-*d*]pyrimidine-2,4-dione antagonist of human luteinizing hormone-releasing hormone (LHRH) receptor containing a urea and versus a methyl ester urea substitution;[148] (B) tryptophan-containing neurokinin-1 (NK-1) receptor antagonists with an *N*,*N*-dimethylaminoethylene moiety at the α-position;[150] (C) substituted aniline enaminone (1) anticonvulsant and the 3-amino isoxazole substitution (2).[151]

ducing an *N*,*N*-dimethylaminoethylene moiety at the α-position that forms an intramolecular H-bond with the amide hydrogen (Figure 19B). Again, no in vitro studies were reported to substantiate the improved permeability or explore whether an efflux pump activity was involved. Last, Eddington et al.[151] reported the introduction of an intramolecular H-bond through an isoxazole decreased active efflux of substituted enaminones (Figure 19C). The isoxazole was markedly more polar and was no longer polarized, but the relative abilities to inhibit P-gp activity were not assessed to rule out that the isoxazoles were inhibiting P-gp at the concentrations tested.

The use of a prodrug approach cannot be ignored as a strategic option to circumvent P-gp.The L-valine ester prodrug of quinidine, a model P-gp substrate, was not polarized in MDCK-MDR1 cells, did not inhibit ritonavir transport, and was not affected by P-gp inhibitor cyclosporin A.[152] These data suggest that L-val-quinidine is not recognized by P-gp. However, active absorptive transport might be a reason for the absence of P-gp efflux since L-val-quinidine might also be recognized by the dipeptide uptake transporter PEPT1, which would circumvent P-gp recognition. A recent review by Vierling and Greiner[153] describes prodrugs of HIV protease inhibitors to improve many of the undesirable physicochemical and pharmacological properties that FDA-approved HIV protease inhibitors have including active efflux. The most successful prodrug is hydrophilic fosamprenavir, a phosphate ester prodrug of amprenavir (Lexiva) that was approved October 2003. Last, other chemical modifications that alter the mechanism by which a drug crosses the biological membrane may also be a means to circumvent P-gp. One example is the addition of a peptide vector to a drug that imparts an ability to traverse the membrane via a nonreceptor-mediated process.[154] When coupled to the P-gp substrate paclitaxel, this vector increased brain uptake.[155]

5. Concluding Remarks

Directly altering the chemistry of a scaffold to circumvent P-gp is possible although only a few published examples exist. And then, the reason for the outcome is not always completely established through the use of multiple assays. For example, there are numerous examples in this review where P-gp circumvention appears to be the result of optimizing a P-gp inhibitor, rather than designing out substrate recognition. The best approach to diminish the limiting effects of P-gp on a particular scaffold is to increase passive diffusion. The efflux pump cannot maintain a gradient and pumping efficiency is poor when passive diffusion is fast enough (section 2). Eliminating groups or substituting with groups that solvate in water (especially H-bond donating groups), or decreasing their H-bonding capacity, and adding halogen groups can increase passive diffusion.[63,156] Reducing

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molecular size, replacing electronegative atoms such as O with S or CH_2, blocking H-bond donors by such means as N-methylating a secondary amine, introducing constrained conformation, and promoting intramolecular hydrogen bonds are all examples of steps to take. The success of each step is likely dependent upon each scaffold and the minimum structural requirement for efficacy and P-gp recognition. The good news is that these changes in passive diffusion also will likely decrease the affinity of binding to P-gp. The bad news is that these alterations may negatively impact intrinsic activity. A change in lipophilicity of a molecule can also have an impact, but the direction of that change depends upon the compound series and its intrinsic physicochemical attributes (section 2). Refinement of the predictive models to deconvolute these multiple contributing factors will advance our ability to drive circumventing SAR more effectively.

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