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Structure and Ligand-based Design of P-glycoprotein Inhibitors: A Historical Perspective

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Abstract: Computer-assisted drug design (CADD) is a valuable approach for the discovery of new chemical entities in the field of cancer therapy. There is a pressing need to design and develop new, selective, and safe drugs for the treatment of multidrug resistance (MDR) cancer forms, specifically active against P-glycoprotein (P-gp). Recently, a crystallographic structure for mouse P-gp was obtained. However, for decades the design of new P-gp inhibitors employed mainly ligand-based approaches (SAR, QSAR, 3D-QSAR and pharmacophore studies), and structure-based studies used P-gp homology models. However, some of those results are still the pillars used as a starting point for the design of potential P-gp inhibitors. Here, pharmacophore mapping, (Q)SAR, 3D-QSAR and homology modeling, for the discovery of P-gp inhibitors are reviewed. The importance of these methods for understanding mechanisms of drug resistance at a molecular level, and design P-gp inhibitors drug candidates are discussed. The examples mentioned in the review could provide insights into the wide range of possibilities of using CADD methodologies for the discovery of efficient P-gp inhibitors.

Keywords: Computer-assisted drug design, homology modeling, P-glycoprotein inhibitors, pharmacophore, quantitative structure-activity relationships, structure-based drug design.

INTRODUCTION

Since the discovery that verapamil could reverse multidrug resistance (MDR) in 1981 [1], a number of studies over recent decades define generic features that are common to compounds which interact with P-gp, through empirical structure-activity relationships (SAR), quantitative structure-activity relationships (QSAR), 3-dimensional quantitative structure-activity relationships (3D-QSAR), or pharmacophore studies. Several homology models of P-gp constructed using prokaryotic ATP-binding cassette (ABC) transporters as templates were also created in order to accomplish the difficult task of understanding P-gp's structure, identifying possible binding sites and screening potential inhibitors. These studies highlight the bottleneck associated with these data sets, such as the unclear distinction of substrates and inhibitors, lack of structural diversity, assay complexities and data inconsistencies.

One of the major mechanisms of MDR is the enhanced ability of tumor cells to actively efflux drugs, leading to a decrease in cellular drug accumulation below effective levels. Active drug efflux is mediated by several members of the ABC super-family of membrane transporters, which are now subdivided into seven families designated from A through G [2]. ABC transporters are present in all organisms and participate in diverse physiological processes. This super-family is composed of more than 100 membrane transporters/channels that are involved in a multiplicity of functions, including the extrusion of harmful compounds, uptake of nutrients, transport of ions and peptides, and cell signaling [3].

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P-gp is perhaps the best characterized human ATP binding cassette transporter. P-gp prevents foreign substances from accumulating in cells by transporting a wide variety of structurally and functionally unrelated compounds from the cell interior into the extracellular space. Energy required for efflux is supplied by ATP hydrolysis [4].Very recently the state of art concerning the described inhibitors of P-gp was reviewed [5]. Herein, we focus mainly on the computational techniques, ligand-based and structure-based, that have been used in the rational drug design of new potential P-gp inhibitors. This review provides an opportunity to organize all the scattered information concerning P-gp modulation, collecting data related to inhibition only. This information, along with recently published structural data for mice P-gp [6], may provide a useful tool for the design of new, more potent and selective P-gp inhibitors.

1. STRUCTURE-BASED DRUG DESIGN

Structure-based approaches to P-gp inhibitors design are based upon an understanding of the molecular recognition between active site groups and interacting molecules. However, a 3D structure of the target is necessary. Until 2009, when a 4.35 Å resolution crystal structure of mouse P-gp was described [6], only low to medium resolution P-gp3D structures had been obtained using cryo-electron microscopy of 2D crystals [7, 8, 9]. Then, a maximum resolution of 8 Å [7, 10] was obtained for a P-gp nucleotide bound form and a resolution of 20 Å for the nucleotide free state [8]. A 8 Å resolution is sufficient to provide a glimpse of the general backbone fold, but tracing the protein main and side chain is not possible, unless through computational interpretations [9].

1.1. P-gp Structure and Potential Binding Sites for P-gp Modulation

Human P-gp is a 170 kDa polypeptide consisting of 1280 amino acids [11] organized in two homologous halves, each encompassing a transmembrane domain (TMD), which contain the drug binding sites and define the translocation pathway across the membrane,

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and one cytoplasmic nucleotide binding domain (NBD), which couple the energy associated with ATP binding and hydrolysis to drug transport [7, 12]. The TMD are homologous with only a small subfamily of ABC transporters, contain the drug-binding sites and form the translocation pathway, while the NBD share a high degree of sequence identity with the equivalent domains of all ABC transporters [13]. Some ABC genes encode proteins that are 'half-transporters' (meaning that two subunits must dimerize to create a functional transporter), whereas others are 'full-transporters'. The ABC transporter subfamilies D and G and 7 members of the B subfamily encode these 4 domains as 2 separate polypeptides (half-transporters). The remaining subfamilies, including P-gp, encode the full transporter as a single multi-domain polypeptide [14].

P-gp inhibitors binding sites are described as the drug binding/efflux site at the TMD, the ATP-binding site at the NBD interface, and the allosteric residues involved in communication pathways [15]. The drug-binding sites are the classic targets for inhibitor design, providing an efficient way to block substrate drugs from being transported (competitive inhibition) [16, 17]. Several P-gp modulators compete as substrates with the cytotoxic agent, for example, verapamil and quinidine for transport by the pump [18]. This diminishes the efflux of the cytotoxic agent, thereby increasing its intracellular concentration. However, the precise pharmacophore features of P-gp inhibitors have yet to be fully characterized due to the plasticity of P-gp drug-binding sites. On the other hand, the NBD may also be an interesting target for the inactivation of P-gp due to the blocking of P-gp's catalytic cycle (noncompetitive inhibition), as described for several flavonoid inhibitors [19]. The distinct motifs contained within the NBD are of great value in *de novo* ligand design [15]. Another promising avenue for the design of inhibitory compounds is presented by those residues identified as being involved in the allosteric communication of drug occupancy [20]. Covalent modification of those residues results in a loss of drug-stimulated ATPase activity [21, 22, 23].

The X-ray structure of apo P-gp at a resolution of 3.8 Å was recently obtained [6] (Table **1**, entry I). It revealed distinct drug-binding sites in the internal cavity capable of stereoselectivity which is based on hydrophobic and aromatic interactions. Apo P-gp structures have an inverted "V" shape, inward-facing conformation, for drug entry, whereas the outward-facing conformation releases the substrate to the extracellular medium [6]. ATP binds to the NBD, causing a conformational change (outward facing) which allows extrusion of the substrate [24]. However, membrane proteins are still hard to make and characterize. The lipids surrounding proteins in cell membranes interfere with the crystallographic procedures, but when not embedded in the lipid bilayer, membrane proteins usually lose their three-dimensional structure [25]. Eventhough, the description of the mice P-gp 3D structure will open new venues for the understanding of the P-gp transport cycle and for the structure-based design of new P-gp inhibitors.

1.2. Homology Modeling

ABC proteins are widespread across all taxonomic groups [54]. Homology modeling is a useful strategy for structure-based drug design when no 3D structural information of P-gp at sufficient resolution is available. A major advance in our understanding of the structure and function of ABC transporters has come from the crystallization and determination of the structures of bacterial transporters [45]. The common feature of all ABC transporters, eukaryotic or prokaryotic, are the TMD, consisting of α-helices embedded in the membrane bilayer, and the NBD, located in the cytoplasm [55]. The structural architecture of ABC transporters consists, minimally, of 2 TMD and 2 NBD [56]. The TMD recognizes a variety of substrates and undergoes conformational changes to transport the substrate across the membrane and its sequence and architecture is variable. Structural studies of ABC transporters suggest that they possess similar large binding sites which may explain their broad substrate specificity and their unusual evolutionary relationships [57]. Since TMD are structurally diverse, some prokaryotic ABC transporters have varying number of helices (Table **1**). The sequence of the NBD is more preserved among different species [57].

Structures of prokaryotic ABC transporters have already provided valuable information on ABC pump structures and on the discovery of new P-gp hit compounds with inhibitory activity through structure-based drug design (Table **1**). Sequence alignments between the prokaryotic template and P-gp target allowed the construction of atomic-resolution homology models, which permitted the design of P-gp inhibitors [43]. They provided molecular detail on how these proteins recognize multiple substrates and helped identify new MDR modulators. Moreover, photoaffinity labeling, cysteine scanning mutagenesis and the use of thiol probes have allowed the identification of the residues involved in the drug binding site located in TMD, which also helped in the validation of some of those models [36].

E. coli's MsbA (Table **1**, entry II), a lipid transporter from *E. coli*, catalyses the transfer of lipids across the cytoplasmic membrane. MsbA crystal structure revealed it as a homodimer adopting a cone shape. It exhibits a 30% homology with human P-gp [58] and is a suitable template for P-gp modeling, as it has a tertiary organization reflecting the accepted consensus NBD dimer interface and consistent with biochemical cross-linking data [36].

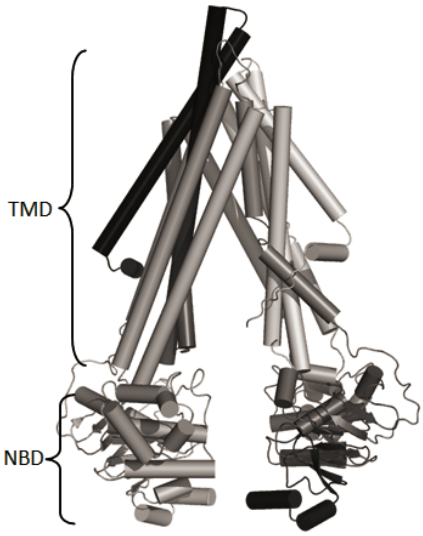
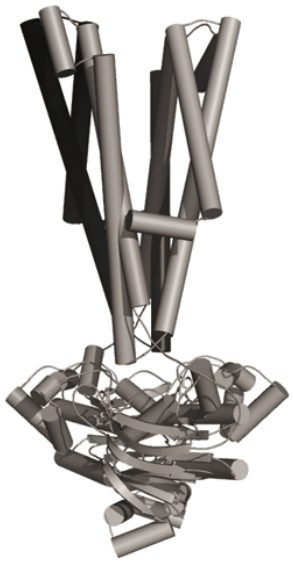
Eventually, the withdrawal of MsbA crystal structures proved a major setback to the field, requiring a reinterpretation of many key pieces of experimental data [59]. The corrected crystal structures of MsbA isolated from several different bacterial species [59] reveal the following three distinct conformations: an inward-facing nucleotide-free conformation, an outward-facing nucleotide-free and a nucleotide-bound form similar to the Sav1866 structure [60]. In the inward-facing form, the NBD are well separated and TMD are in contact only near the periplasmic surface of the membrane, creating a V-shaped molecule. The large variations between the three structures suggest that substantial conformational changes may take place during the catalytic cycle of an ABC transporter [56]. The newly described structure of MsbA allowed its convenient use as a template for P-gp homology modeling in the following years [31, 32].

The crystal structure of the prokaryotic homologue Sav1866 (Table **1**, entry III) provided a new opportunity for the construction of new P-gp models [60]. The two TMD form a chamber in the membrane which is open extracellularly (in the ATP bound state). The two NBD have an ATP-sandwiched dimer conformation [61]. The organization and pseudo-symmetrical arrangement of TMD on P-gp [7] correlates with the TMD arrangement of Sav1866, and the overall dimensions of the two transporters also correspond. Besides, P-gp possesses the same domain inter-linking motif as Sav1866, where P-gp intracellular loop 4 (ICL4) interacts with residues in NBD1 [62], strengthening the argument for Sav1866 use as a suitable template structure on which to model P-gp.

BtuCD (Table **1**, entryIV) is an ABC transporter for the uptake of vitamin B12 in *E. coli* [45] and is composed of two subunits. BtuCD has better resolved NBD but non-homologous TMD (20 TM segments compared to P-gp's 12) in its structure. In contrast to MsbA, the tertiary structure of Btu CD contains a parallel TMD: TMD interface [63]. Although biochemical evidence and structural data clearly show P-gp to be an asymmetric molecule, homology models of P-gp built using the crystallographic structures of BtuCD as templates show a symmetric structure [7].

Maltose transporter $MalFGK_2$ was also used as a template for homology modeling of the ATP-binding site of P-gp [43]. Three-dimensional models of human P-gp NBD were also built by homology modeling using high-resolution ABC transporters TAP1 [49, 50], HisP [51], Mj0796 [36], HlyB [52] or Rad50 [36] (Table **1**, entries VI-X) structures as templates, allowing interactions between NBD and ligands to be investigated using *in silico* docking studies,

Table 1. Transporters used as P-gp Homology Modeling Templates

	Template	Source	Structure	Activity	Homology Modeling Studies
I	**P-gp** 	Mouse (PDBID: 3G60* and 3G61)	Full-transporter: 2 TMD (each with 6 TM) and 2 NBD [26]	Multidrug export [26]	Analysis of antifouling biocides binding site [27] Differences in the activity of diastereoisomers of benzopyrano [3,4-b] [1,4]oxazines [28] Identification of binding hypotheses for propafenone-type P-gp inhibitors [29]
II	**MsbA** 	*Salmonella typhimurium* (PDBID: 3B60* and 3B5Z) *Escherichia coli* (PDBID: 3B5W) *Vibrio cholerae (*PDBID: 3B5X)	Homodimer of half transporters, each subunit containing a TMD (6 TM helices) and a NBD [30]	Lipid A export [30]	P-gp homology model based on a wide open inward-facing conformation of *E. coli* MsbA. Amino acids Ile306 (TM5), Ile340 (TM6), Phe343 (TM6), Phe728 (TM7), and Val982 (TM12) form a putative substrate recognition site in the P-gp model, which is confirmed by both the P-gp X-ray crystal structure and the site-directed mutagenesis studies [31] Interpretation of the effects of several mutants in the NBD, within the TMD or at the NBD:TMD interface [32] Comparisons between MsbA structure and P-gp model reveals mutations on P-gp 'hot-spots' that alter specificity of binding [33] Analysis of substrate binding domains [34] Analysis of models of two different functional states of P-gp corresponding to nucleotide-free and nucleotide-bound P-gp [35] TMD of MsbA are reoriented with respect to the NBD to create a P-gp model [36] Model of P-gp open conformation [37] Study the reversal of P-gp mediated MDR by (-)-epigallocatechingallate and its molecular mechanism [38] Creation of an atomic scale model of P-gp [36]

(Table 1) Contd....

	Template	Source	Structure	Activity	Homology Modeling Studies
III	**Sav1866** 	*Staphylococcus aureus* (PDBID: 2HYD* and 2ONJ)	Homodimer of half transporters, each subunit containing one TMD with six helices and one NBD [39]	Multidrug export [39]	Design of P-gp model for drug delivery using protein hydration thermodynamics [40]
					Analysis of Sav1866 as a well-defined model for studies on the molecular bases of drug-protein interactions in ABC transporters [41]
					Identification of putative binding sites on P-gp [42]
					Construction of an ATP-bound outward facing model of P-gp using the Sav1866 crystal structure as a template, and comparison with a previous MRP5 model [24]
					P-gp models of the apo and ATP-bound states based on homology with Sav1866 and MalK [43]
					P-gp model used to screen for virtually designed molecules as potential P-gp inhibitors [44]
IV	**BtuCD** 	*E. coli* (PDBID: 1L7V* and 2QI9)	Two NBD (BtuD) are in close contact with each other, as are the two TMD (BtuC). Each TMD has 10 transmembrane helices grouped around a translocation pathway that is closed to the cytoplasm by a gate region. BtuF is the periplasmic binding protein [45]	Uptake of vitamine B12 [45]	Creation of an atomic scale model of P-gp [7, 36]

(Table 1) Contd....

	Template	Source	Structure	Activity	Homology Modeling Studies
V	**$MalFGK_2$** 	*E. coli* and *Thermococcus litoralis* (PDB ID: 3FH6, 2R6G*)	The membrane transporter $MalFGK_2$ is made up of two integral membrane proteins (MalF and MalG) and two copies of the ATP-hydrolyzing subunit (MalK), and aperiplasmic maltose binding protein (MBP) [46]	Uptake of maltose [47]	P-gp models of the apo and ATP-bound states based on homology with Sav1866 and MalK [43]
VI	**TAP1 ****	Human endoplasmic reticulum resident protein (ATP binding site only; PDBID: 1JJ7)		Transports cytosolic peptides generated by the proteasome to the ER lumen for loading onto MHC class I molecules [48]	Model interactions between P-gp and flavonoids [49] Investigate the molecular mechanism involved in ATP binding and hydrolysis [50]
VII	**HisP** **	*S. typhimurium* (ATP binding site only; PDBID: 1B0U)		Histidine permease [51]	Binds ATP analogs similarly to the mammalian P-gp, suggesting that ATP coupling is a common mechanistic basis for the membrane-associated function of these proteins [51]
VIII	**Mj0796** **	*Methanococcus jannaschii* (ATP binding site only; PDBID: 1F3O and 1L2T)		LolD transporter [36]	Creation of an atomic scale model of P-gp [36]
IX	**HlyBprotein ****	*E. coli* (ATP binding site only; PDBID: 2FF7, 1XEF, 1MT0)		Haemolysin A exporter [52]	P-gp viewed as a tandem duplication of the HlyB protein [52] Analysis of the interactions of 5′-fluorosulfonylbenzonyl 5′-adenosine , an ATP analogue, with P-gp to study the catalytic cycle of ATP hydrolysis [53]
X	**Rad50 ****	*Pyrococcus furiosus* (ATP binding site only; PDBID: 1F2U, 1F2T,1II8,1US8)		DNA repair [36]	Creation of an atomic scale model of P-gp [36]

*structure represented on the image; **only the ATP-binding site structure is available

or using MD simulations to identify conformational changes in P-gp.

The factors that determine the quality of a homology modeling study are the percentage of identity between the protein of interest and the template (which should be greater than 25-30 %), and the quality of the crystal structure used as the template. There are several ways for improving the accuracy of the sequence alignment, such as alignment with multiple templates. However, for the majority of transmembrane proteins the choice of template structures is a critical limitation. Unfortunately, templates differ from P-gp in

structural organization. It must be noted that a homology model is an approximation of the structure and the exact positioning of the various domains may differ from the target. Comparison with experimental data may provide more reliable models.

2. LIGAND-BASED DRUG DESIGN

P-gp ligand-based drug design relies on knowledge of compounds that are known to inhibit that transporter and represents an useful CADD strategy when the structure of the target is not available [64]. Structure activity relationship (SAR) [65], quantitative structure-activity relationship (QSAR) [66], and three-dimensional quantitative structure activity relationship (3D-QSAR) [67], may be used to predict the activity of new analogs. A pharmacophore model [68] defines the minimal structural features amolecule must possess in order to bind to P-gp. These are the ligand-based strategies most frequently used.

2.1. (Q)SAR Studies of P-gp Inhibitors

The amount of literature involving the SAR of P-gp is huge, complex and dates back several decades. Since the discovery of verapamil as a MDR reversing agent in vincristine resistant P388 leukaemia *in vivo* and *in vitro* [1], several SAR and QSAR studies (Table **2**) were published by several work groups. One of the first structure-activity relationship studies concerning MDR modulation was performed in 1988 using a series of vinca alkaloids with diverse structures and properties. The authors concluded that lipidic solubility at pH 7.4 and the presence of the molecule in a protonated form were important features for MDR reversal activity [69] (Table **2**, entry 1).

SAR studies examine empirically how structural alterations of individual molecules (with concomitant alteration of physicochemical features) influence their ability to inhibit P-gp. On the other hand, QSAR studies quantify the observed relationship between molecular descriptors and activity. QSAR relates numerical properties of the molecular structure to its activity via a mathematical model [102]. A structure-activity relationship study can help to establish which features are implied in the activity and help to orient the synthesis towards the structural modifications which would enhance the activity. The major achievements concerning P-gp inhibition (Q)SAR studies from 1988 until now are presented in Table **2**.

MDR modulation is a complex process and many different pathways can contribute to it. Besides, until now, there has not been a complete understanding of the molecular mechanisms involved in P-gp inhibition. These facts raise some problems on QSAR studies of P-gp modulators [103]. For more than two decades QSAR studies have been one of the rational approaches to understand the binding affinities of several classes of compounds towards P-gp. Multiple linear regression, principal component regression, and partial-least squares regression are frequently used statistical methods [104].

Several critical assumptions can influence the validity and correctness of any QSAR study. For example, the molecules under study must act through the same mechanism, must share the same binding mode to P-gp and biological results must efficiently correlate the biological activity with the binding affinities [105]. Meeting these assumptions ensures that proper and reliable relationships are obtained. Besides, most QSAR studies deal with a limited number of molecules (Table **2**, entries 1, 2-7, 9, 13, 16, 20, 28, 31-33), and few published studies give activities for sets of compounds with sufficient molecular diversity [106]. Therefore, although useful in the process of compound development, QSAR studies are not always applicable to other structural series.

In some cases simple molecular descriptors such as partitioning coefficients or molecular weight and simple models such as multiple linear regressions can establish a QSAR. However, in other situations, complex molecular descriptors and methods like artificial neural networks and genetic algorithms are needed to establish a relationship between structure and P-gp inhibitory activity [107].

Although many studies provide data (Table **2**) that could be used to develop (Q)SAR, this data is often not directly comparable. Literature is replete with examples of several types of biological activity data, such as: cell growth; efflux assays; direct effects on membrane properties; effects on rates of transport; levels of ATPase activity; and many others. Important issues that need to be considered include: the underlying mechanism probed by the assay; binding site; cell line; approach used to induce expression of P-gp; membrane partition coefficients; and permeation rates of the individual compounds [108]. The measured compound's effect on P-gp activity does not necessarily correlate with its "binding" affinity to P-gp, and it has not been established that binding must confer activity, which makes data analysis even more complex [91]. Moreover, assays might target different mechanisms of action (competition with a substrate, inhibition of the ATPase function, or blockage of the transporter). Even if the same mechanism is targeted by an assay, results could differ. Nonetheless, MDR reversal effects of the same compounds in different cell lines, or in similar cell lines from different species, and the use of different cytotoxic agents can lead to different results [109]. Biological results should be carefully assessed before being used in an SAR study.

Let us focus on the descriptors that are frequently used in (Q)SAR studies (Table **2**). LogP of the compound or lipophilicity of substituents appears in several SAR (Table **2**, entry1, 2, 4, 6, 12, 14-16, 20, 22, 24, 27, 28, 32, 34-37). However, different approaches and programs were used to calculate these values. Molecular weight (Table **2**, entry 14, 24-27), number of H-bond donors and acceptors (Table **2**, entry 14, 18, 21, 23, 30, 37), number of aromatic rings (Table **2**, entry 7, 10, 15, 21), cationic charge (Table **2**, entry 1, 2, 8), such as a protonable amine (Table **2**, entry 3, 6, 8, 9, 12, 17, 2), and type and position of substituents (Table **2**, entry 6, 10, 11, 17, 19, 21, 29, 31-33, 36) are described as being implied in P-gp inhibition.

2.2. 3D-QSAR

3D-QSAR refers to the application of molecular field calculations requiring 3D structures. It examines the steric fields (shape of the molecule) and the electrostatic fields based upon an energy function, correlating them to P-gp inhibitory activity of the compounds [67, 110]. A comparative molecular field analysis (CoMFA) and a comparative molecular similarity index analysis (CoMSIA) are the most frequent 3D-QSAR algorithms [111]. Examples of 3D-QSAR are listed in Table **3**. In 1997, the first 3D-QSAR analysis using structurally related thioxanthenes, was performed [112] (Table **3**, entry 1). Results show that the 3D location of electrostatic and steric fields, as well as lipophilic and hydrophilic regions, in both enantiomerscoincides with the presence of certain groups already described in section 2.1. such as tertiary nitrogen atoms, aromatic rings, H-bond donors and acceptors which influence the P-gp inhibition activity.

2.3. Pharmacophore Modeling

Pharmacophore is an abstract description of molecular features which are necessary for molecular recognition of a ligand by a biological macromolecule. The first attempt to characterize important pharmacophore features for P-gp modulation was performed by Seelig in 1998. Seelig suggested a pharmacophore based on specific arrangements of electron-donor groups [120] (Table **4**, entry 1).

Considering that the P-gp protein accommodates a variety of compounds with different structures, and their binding sites and modes are not well known, it is not surprising that a consensus set of features may not exist. Several pharmacophore models (Table **4**) have been described in the literature to retrieve P-gp inhibitors with considerable accuracy [68, 123, 128]. However, as for the (Q)SAR studies, most of the pharmacophore models are limited to a small

Table 2. SAR and QSAR Studies Concerning P-gp-mediated MDR Reversal

Entry	Compounds	Method	Conclusion
1	Several vinca alkaloids P-gp modulators	SAR	Lipidic solubility at pH 7.4 and presence of a protonable group favour MDR reversal activity [69]
2	24 Structurally diverse substances and verapamil	SAR	Physicochemical properties, namely lipid solubility at physiological pH, cationic charge and molar refractivity, are important for anti-MDR activity of the studied modulators [70]
3	Reserpine, yohimbine and nine derivatives	SAR	Benzoyl and especially a 3,4,5-trimethoxybenzoyl substituent, increased MDR modulating activity; two planar aromatic domains and a distant basic nitrogen also hypothesised as important for MDR reversing activity; lack of a correlation with log P [71]
4	Series of dihydropyridines	SAR	Lipophilicity of the molecules was the main determinant for their MDR reversing activity [72]
5	Hundred MDR reversing-compounds	SAR	Several parameters simultaneously account for anti-MDR activity, making it difficult to establish a direct relation of the obtained results to MDR activity [73]
6	Series of phenothiazines and structurally similar drugs	SAR	A lipophilic electronegative group (Cl, CF_3) in position 2 of the tricyclic ring system enhances anti-MDR activity, while a hydroxyl group decreases it; the tertiary amines are more potent than primary or secondary amines and the derivatives with a piperazine moiety are the most potent ones; *N*-methyl substituted piperazines are better than *N*-hydroxyethyl; activity increases with increasing chain length between the nitrogen and the tricyclic ring system [74]
7	Phenothiazines and related-drugs	SAR	Aromatic rings in P-gp are oriented by the α-helix conformation in such way as to overlap the πorbitals of the aromatic groups in the phenothiazines and thioxanthenes. The hydrophilic acidic residues in P-gp interact with the positively charged amino side of the drugs [74]
8	Drugs belonging to a wide variety of pharmacological classes as calcium channel blockers, neuroleptics, antiarythmics, antimalarials, antiestrogenes, antineoplastics, and others	SAR	The active MDR reversing agents were both hydro- and lipophilic, protonable, and all had an aromatic structure and a basic amino alkyl group [75]
9	Eight compounds with different tricyclic ring systems without a basic side chain	SAR	Electronegativity of the heteroatom bound to the tricyclic ring system and the presence of a NH-group [76]
10	232 Phenothiazines and related drugs	SAR	Among the potent reversers, many contained a carbonyl group, a feature not described by other authors. Other features were the lack of amino group, the presence of a carboxyl group, the presence of a single ring structure or the situation in which one or both rings are of the pyridine type [77, 78]
11	311 Structurally diverse compounds possessing two or more phenyl rings	SAR	Permanent charge as in quaternary amines abolished activity while high anti-MDR activity required the presence of two or three phenyl rings. The rings could be spatially close and connected by one alkyl bridge with a secondary or tertiary amine group or distant and connected by two alkyl chains (as in verapamil). The presence of a carbonyl group was found to increase anti-MDR activity and/or a dimethoxyphenyl function was beneficial [78]
12	70 Derivatives of almitrine	SAR	The length of the spacer between the triazine ring and the lipophilic moiety was the most important for the reversal effect; a hydrophobic aromatic domain and two amine groups, one of which protonated at physiological pH were also important [79]
13	14 Verapamil analogs	SAR	No difference in MDR reversal activity when the methoxyl groups in the phenyl rings were replaced by chlorine, or when one phenyl ring was replaced by an alkyl chain. Replacement of the methoxyl groups by hydrogens slightly reduced activity [80]
14	Series of pesticides	SAR	Both inhibitors and substrates: at least one 6-membered cyclic structure MW of the substrates > 399 and of the inhibitors > 247 Log P of substrates < 2 H-donor potential of substrates > 0.25 Dipole moment of inhibitors >3.3 [81]

(Table 2) Contd....

Entry	Compounds	Method	Conclusion
15	24 Quinoline type compounds	SAR	In the hydrophobic moiety the aromatic rings were essential; the most active compounds possessed two aryl rings with a nonplanar arrangement (related to the possibility of π-hydrogen-πinteractions if hydrogen bond donors of P-gp were set between two deviated aromatic rings). Compounds with phenyl ring not substituted with chlorine or fluorine were more potent [82]
16	Series included guanidinum and pyridinum derivatives	SAR	At least one aromatic moiety and certain degree of lipophilicity (log P > -1) were necessary for MDR activity [83]
17	609 Compounds of diverse structures	SAR	Importance of a dialkyl-substituted amine; quaternary amines did not necessarily enhance activity; the arrangement of the alkyl groups appeared to affect the activity; molecules possessing a *cis*arrangement and a nitrogen in the ring were less often active than when at least one of the alkyl chains was acyclic [84]
18	22 Diverse drugs	QSAR	An inverse relationship was established between surface area and P-gp ATPase activity. The number of hydrogen bond acceptors and estimated strength of these were also found significant [85]
19	21 2-Chloro-10-substituted phenoxazines	SAR	Chlorine atom at position 2 and introduction of an alkyl side chain containing a tertiary amino group at a distance of at least three to four carbon atoms from the N10 are favourable for MDR activity [86]
20	Series of halogenated chalcones	SAR	Flavone-type compounds are assumed to interact with the ATP binding site and therefore are not transported by Pgp. Lipophilicity of the ring substituents was related with MDR reversal activity [87]
21	59 Tetrahydroisoquinolines and isoindolines	SAR	Electron donating alkoxy substituents on the two aromatic rings positively correlated with MDR reversal. Length of the linker connecting the two aromatic rings or the replacement of the cyano group by hydrogen did not affect MDR modulation [88]
22	28 Flavonoid derivatives containing a *N*-benzylpiperazine chain	SAR	Very hydrophilic compounds were inactive; position of hydroxyl groups affected MDR reversal [89]
23	100 Substrates/inhibitors obtained from the literature	SAR	Specific arrangements of hydrogen bonding moieties [65]
24	Substrates and modulators as well as clinically promising MDR reversing agents	QSAR	MDR-reversal activity was correlated with the lipophilicity (LogP) and molecular weight (MW) [90]
25	Diverse set of 22 drugs	QSAR	Activity related to molecular size and polarity [66]
26	157 Phenothiazines	QSAR	Good correlation was obtained with descriptors that model molecularsize and polarizability. Hydrogen bonding or hydrophobicity did not play a role in MDR reversal activity [66]
27	Diverse set of P-gp modulators	QSAR	A highly effective P-gp modulator candidate should possess a log P value of 2.92 or higher, 18-atom-long or longer molecular axis, and a high E_{homo}value, as well as at least one tertiary basic nitrogen atom [91]
28	Jatrophane polyesters	SAR	P-gp inhibitory activity increases with lipophilicity. The substitution patterns affects MDR reversal activity [92]
29	5,7,3',4',5'-Pentamethoxyflavone (PMF) and derivatives	SAR	Methoxyl derivatives and numbers or positions are more important than their hydroxylated counterparts in chemosensitization [93]
30	78 Inhibitors from the literature	QSAR	Mean values of number of hydrogen bond acceptors and donors of 1.7 and 6.7, respectively, were found for inhibitors. Substrates tend to have a higher number of H-bonds than inhibitors [94]
31	Series of *N*-acyloxy-1,4-dihydropyridines	SAR	Methoxyl groups within the phenyl moiety lead to highest activity; regiospecific effects on the observed activities indicated a conserved interaction between P-gp inhibitor function and P-gp binding region [95]

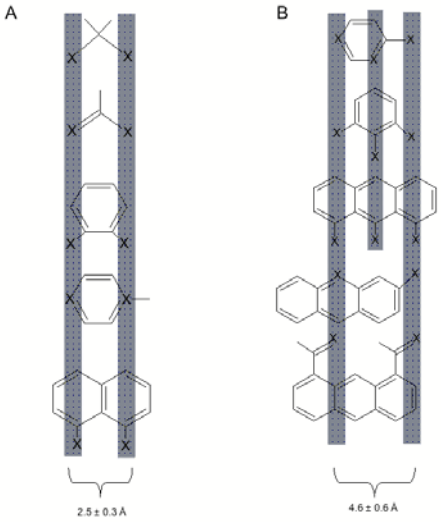
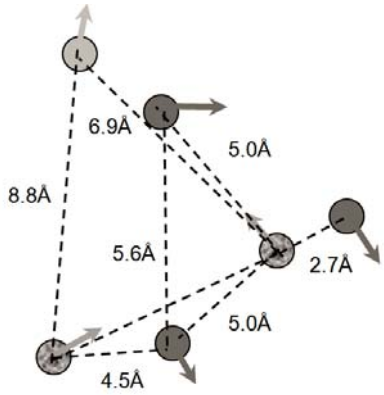
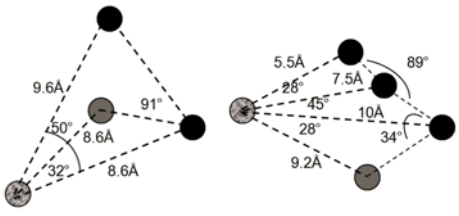
(Table 2) Contd....

Entry	Compounds	Method	Conclusion
32	3,9-Diazatetraasteranes with varied aromatic substitution patterns	SAR	Increased lipophilicity was found favourable. Highest activities were found for *meta* substituted compounds (regiosensitivity of the potential P-gp binding site) [96]
33	New pyranocoumarins	SAR	The co-existence of 3- and 4-methoxyl groups remarkably enhanced the Pgp-inhibitory activity; the lone existence of the 4-methoxyl group reduced the activity [97]
34	Dataset of the Prestwick Chemical (library retrieved from PubMed)	QSAR	Lipophilicity influences P-gp modulation [98]
35	Library of flavonoid homodimers and heterodimers	SAR	Flavonoid dimers with nonpolar and hydrophobic substituents (methyl and ethyl groups) were more potent MDR reversers than dimers with polar and hydrophilic substituents (hydroxy groups). Bulkier substituents lead to lower reversing activity [99]
36	22 Flavonoids	SAR	Structural units of B-ring-3'-OH group, B-ring-4'-OH group, C-3-ring (or structural skeleton of isoflavones), and logD negatively contributed to the modulation effect of flavonoids on P-gp activity, while the A-ring-7-OH group tended to enhance their inhibitory effects [100]
37	772 Diverse compounds	QSAR	Hydrophobic surface area, LogP, and descriptors of size (such as molecular surface), flexibility, hydrophilic volume, distance between two hydrophobic atoms and one hydrogen bond acceptor atom were important for P-gp inhibitory activity [101]

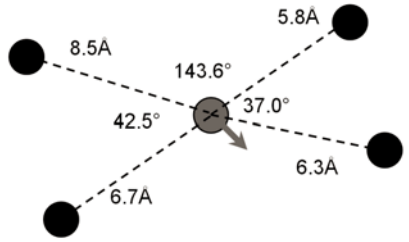
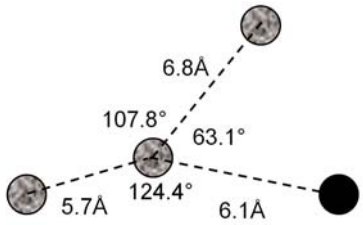
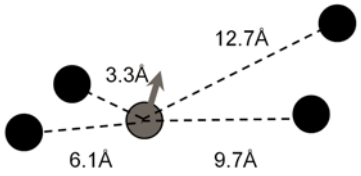
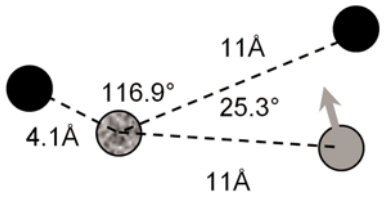
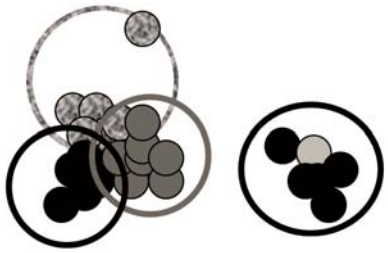
Table 3. 3D-QSAR Studies Concerning P-gp-mediated MDR Reversal

Entry	Molecules	3D-QSAR Method	Result
1	Thioxanthene analogs	CoMFA	Electrostatic, hydrophobic and lipophilic fields of enantiomers are differently positioned in the 3D space, which justifies the different P-gp modulatory activity [112].
2	Phenothiazines and related drugs	CoMFA	Best models either with hydrophobic fields alone or in combination with steric and electrostatic fields pointing to hydrophobicity as a property of primary importance [113].
3	46 Imidazoles	CoMFA and CoMSIA	A large substituent on the imidazole ring is not favoured for improving MDR modulating potency [67].
4	Dihydro-β-agarofuran sesquiterpene derivatives	CoMSIA	Provided information on hydrogen bond donor and acceptor requirements of sesquiterepenes; substituents at the C-2 position act as hydrogen bond acceptor; the oxygen of the furan ring seems to form a hydrogen bond with the receptor [114].
5	32 Natural and synthetic coumarins	CoMFA	Favorable electrostatic and steric volumes, like the α-(hydroxyisopropyl) dihydrofuran moiety, beside C(5)-C(6) or C(7)-C(8) positions. An important hydrophobic, neutral charge group, like phenyl, in position C(4) on the coumarinic ring [115].
6	76 Dihydro-β-agarofuran sesquiterpenes	CoMSIA	The most important features are the substituents at the C-2, C-3, and C-8 positions, which seem to be critical for determining the overall effectiveness of sesquiterpenes as P-gp inhibitors [116].
7	24 Structurally related derivatives of tariquidar	CoMFA and CoMSIA	3D-QSAR with an internal predictive squared correlation coefficient higher than 0.8; included electrostatic, steric, hydrogen bond acceptor, and hydrophobic fields. The best single field model was the electrostatic one, followed by the steric and hydrophobic. The combination of the electrostatic with the steric, hydrophobic, and acceptor indices yields the models with best statistical characteristics [117].
8	Third generation MDR modulators	CoMFA	Presence of tertiary nitrogen, a central phenyl ring and an aromatic dimethoxy group contributed to the inhibitory effect [118].
9	41 Flavones	CoMFA and CoMSIA	Hydrophobic and steric parameters are important for P-gp inhibitory activity [119].

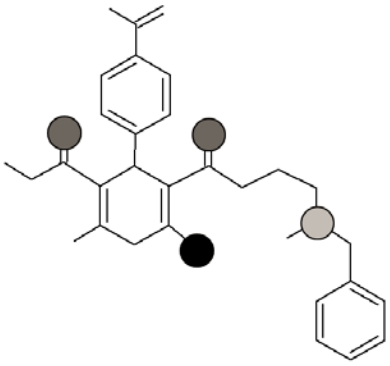
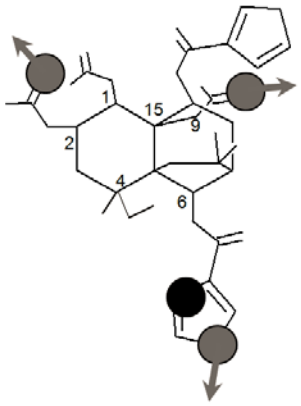
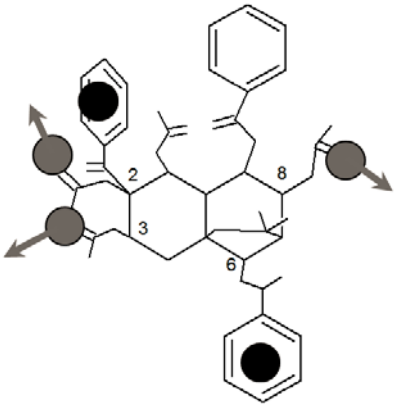
Table 4. Pharmacophores for P-gp inhibitory Activity

Entry	Molecules	Method / software	Features important for activity	Pharmacophore (black= hydrophobic group; dark grey= hydrogen bond acceptor; light grey= hydrogen bond donor; spotted= aromatic ring; white= positive charge group)
1	Inducers and inhibitors of P-gp with SAR studies having 100 compounds as input taken from literature	Measurement of the distances between electron donor groups (X)	Two pharmacophore models for P-gp modulators: - Type I is two electron donor groups separated by 2.5±0.3 Å. - Type II contains two electron donors separated by 4.6± 0.6 Å, possibly with a third electron donating grouping between outer two. All the compounds with at least one of these features were found to be P-gp modulators. Most of them contain at least two of these pharmacophores, some contain as many as eight [120].	 Electron donor patterns observed in P-gpmodulators. (A) Patterns formed by electron donor pairs with a spatial separation of 2.56±0.3Å. (B) Patterns formed either by three electron donor groups with a spatial separation of the outer two electron donor groups of 4.66±0.6Å , or by two electron donor groups with a spatial separation of 4.66±0.6Å (adapted from [120])
2	A variety of drugs that are P-gp modulators	GASP (genetic algorithm similarity program)	Two aromatic rings, three hydrogen bond acceptor groups and one hydrogen bond donor group [68].	 General pharmacophore pattern of drugs at the verapamil binding site of P-gp (adapted from [68])
3	Various cyclic peptide compounds	Pharmacophore (Molcad)	Two different, but partially overlapping, pharmacophores were described. Pharmacophore A binds verapamil, cyclosporine A, and actinomycin D and contains one aromatic area, two alkyl areas (hydrophobic), and one hydrogen bond acceptor group. Pharmacophore B binds vinblastine and contains one aromatic area, three alkyl areas (hydrophobic), and one hydrogen bond acceptor group. These pharmacophores 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 [121].	 Schematic representation of the pharmacophores, with characteristic angle and distance values connecting the pharmacophoric points in the space. Drug molecular recognition by P-gp, as deduced from molecular modeling data for verapamil, cyclosporin A, actinomycin D, bromocriptine, and pristinamycin I_A (pharmacophore A) and for vimblistine and tentoxin (pharmacophore B) (adapted from [121]).

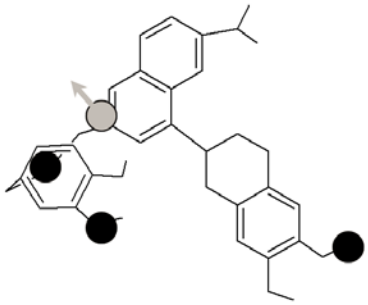
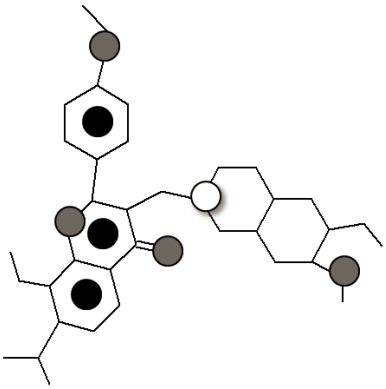
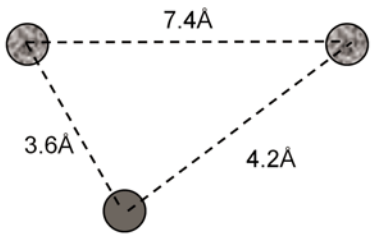
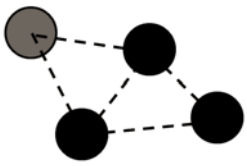
(Table 4) Contd....

Entry	Molecules	Method / software	Features important for activity	Pharmacophore (blue= hydrophobic group; green= hydrogen bond acceptor; purple= hydrogen bond donor; orange= aromatic ring; red= positive charge group)
4	27 Inhibitors of digoxin transport in Caco-2 cells	Pharmacophore (Catalyst)	Four features: four hydrophobes and one hydrogen bond acceptor (non-basic amines that have a lone pair, *sp* or *sp2* nitrogens, *sp3* oxygens or sulfurs, and *sp2* oxygens) [122].	 The inhibition of digoxin transport P-gppharmacophore (adapted from [122])
5	21 Inhibitors of vinblastine accumulation in vesicles derived from-CEM/VLB100cells	Pharmacophore (Catalyst)	Three aromatic ring features and one hydrophobic feature [122].	 The inhibition of vinblastine transport P-gp pharmacophore showing features and distances (adapted from [122])
6	17 Inhibitors of vinblastine accumulation in P-gp expressing LLC-PK1 cells	Pharmacophore (Catalyst)	Four hydrophobes and one hydrogen bond acceptor [122].	 Inhibition of vinblastine accumulation P-g pharmacophore (adapted from [122])
7	22 Inhibitors of calcein accumulation in P-gp expressing LLC-PK1 cells	Pharmacophore (Catalyst)	Two hydrophobic groups, an aromatic ring feature, and a hydrogen bond donor [122].	 Inhibition of calcein accumulation P-gp pharmacophore (adapted from [122])
8	Pharmacophore merging	Pharmacophore (Catalyst)	Clusters of identical features such as hydrophobes, hydrogen bond acceptors, and ring aromatic features. These findings suggested that vinblastine, verapamil, and digoxin have an overlapping affinity for similar or identical binding site(s) within P-gp [122].	 Merged P-gp inhibition pharmacophores defining common areas of hydrophobicity, hydrogen bonding, and ring aromatic (adapted from [122]).

(Table 4) Contd....

Entry	Molecules	Method / software	Features important for activity	Pharmacophore (black= hydrophobic group; dark grey= hydrogen bond acceptor; light grey= hydrogen bond donor; spotted= aromatic ring; white= positive charge group)
9	195 P-gp substrates and inhibitors	Pharmacophore (CONAN)	Amphiphilic nature of P-gp modulators. The pharmacophore includes combinations of hydrophobic or aromatic groups, hydrogen bond acceptors, and hydrogen bond donors. Four point pharmacophores are typically the dominant element of this study.	 Example of a four-point pharmacophore mapped onto conformations of nicardipine scaffold (adapted from [123])
10	Dihydro-β-agarofuran sesquiterpene derivatives	Pharmacophore (CoMSIA)	A four-feature pharmacophore with three hydrogen-bond acceptors and one hydrophobic group was established. Important features are the carbonyl group of a benzoate, acetate, propanoate, and methylbutyrate substituents at the C-2 position that act as a H-bond acceptor depicted as a green sphere. The oxygen of the furan ring at the C-6 position seems to form a hydrogen bond with the receptor (dark grey sphere) [114].	 Four-point feature pharmacophore (adapted from [114])
11	Dihydro-β-agarofuran sesquiterpene derivatives	Pharmacophore (CoMSIA)	Carbonyl groups at the C-2, C-3, and C-8 positions act as a H-bond acceptor depicted as green spheres. A bulky hydrophobic substituent at the C-2position is depicted as a dark grey sphere. A hydrophobic substituent at the C-6 position is depicted as a black sphere [116].	 Five-point feature pharmacophore (adapted from [116])

(Table 4) Contd....

Entry	Molecules	Method / software	Features important for activity	Pharmacophore (blue= hydrophobic group; green= hydrogen bond acceptor; purple= hydrogen bond donor; orange= aromatic ring; red= positive charge group)
12	33 P-gp inhibitors (activity range, 0.024–100 μM)	Pharmacophore (catalyst)	Three hydrophobic features and one hydrogen bond donor features [124].	 Four-point feature pharmacophore (adapted from [124])
13	Quinazolinones, indolo- and pyrrolo-pyrimidines	Pharmacophore (GALAHAD implemented in SYBYL)	A eight point feature pharmacophore (four hydrogen bond acceptor groups, three hydrophobic regions and one positively charged group) was established [125].	 Eight-point feature pharmacophore (adapted from [125])
14	26 Flavonoids	Pharmacophore (PharmaGist)	Two aromatic rings and one hydrogen bond acceptor feature [126].	 Three feature pharmacophore for flavonoid P-gp inhibitors (adapted from [126])
15	Macrocyclic diterpenes	Pharmacophore (MOE)	Three hydrophobic regions and one hydrogen bond acceptor group [127].	 Four-point feature pharmacophore (adapted from [127]).

number of compounds and do not directly define the P-gp binding site for which they are directed [108].

Regarding pharmacophore features, the presence of one or two hydrophobic centres, including one or two aromatic rings, one to three H-bond acceptors (oxygen in an amide, hydroxyl or carbonyl group), and/or one H-bond donor (H in an amine or hydroxyl group) are hypothesized as being the most relevant for P-gp inhibition (Table **4**). Features may vary due to the different compound training sets, biological assays, and software used.

A distinction between competitive and noncompetitive P-gp inhibitors has been achieved by our group, using *in vitro* ATPase results obtained for thioxanthonic derivatives [44] and several

commercially available drugs [126] obtained using the same protocol and the same conditions (unpublished results). Two 3D-pharmacophoric model were created using HypoGen module of Catalyst program [127, 128] according to the results obtained in the ATPase assay for twenty three noncompetitive and for nineteen competitive inhibitors both newly synthetized thioxanthonic derivatives and commercial drugs previously described [42, 124]. Despite all the progress that has been made in the field of pharmacophore construction, the establishment of a consensus pharmacophore for P-gp inhibition has not been achieved so far. These studies have helped to unveil the pharmacophore features responsible for biological activity. However, as data continues to increase, a more detailed understanding of the P-gp structure allows a better understanding of the causes for the variability in the biological results available nowadays.

3. IMPORTANT P-GP INHIBITORS DESCRIPTORS / FEATURES

Summarizing the results of the above reviewed studies, one can postulate several structural requirements for compounds involved in P-gp associated MDR reversal. The molecular descriptors that have been used to describe the properties of P-gp modulators can range from simple constitutional descriptors like number of atoms, bonds or rings, functional group descriptors such as number of H-bond acceptors and donors and amine groups, molecular property descriptors such as logP, to 3D features or more complex quantum chemical descriptors that describe the electrostatic and electronic properties of the molecule.

No general, valid common structural pattern can be formulated. The MDR modulators are so structurally diverse that it is difficult to identify the common structural elements they share. It should be noted that many of the descriptors used are correlated to each other. For example, molecular weight, surface area and volume are all related. For drug molecules, lipophilicity tends to increase with size. Lipophilicity and the number of H-bond donors and acceptors would be expected to correlate inversely.

In general, substrates have a lower logP than the inhibitors, some of them relatively hydrophilic too. Many studies have found that the H-bond acceptor groups are important elements of P-gp substrate recognition [91]. P-gp inhibitors act predominantly as H-bond donors rather than H-bond acceptors [81]. Substrate binding and P-gp activation increase with the number of H-bond acceptor units [65].

Steric or shape complementarity between the ligand and receptor has always been thought as an important factor for effective drug-receptor interactions. The most elemental characterization of the molecular size, the molecular weight shows that small molecules (MW < 250 $g.mol^{-1}$) cannot be P-gp substrates and inhibitors. The range of appropriate MW varies from about 250 to 2000 $g.mol^{-1}$ [129], and it can be further reduced to 250 – 500 $g.mol^{-1}$ when considering Lipinski's rules [130].

P-gp modulators display lipid solubility at physiological pH, planar appearance, and cationic charge usually in a nitrogen-containing cyclic ring [70]. These features were described more than two decades ago and are still used in drug development. A more recent set of criteria was described for P-gp modulators (Fig. **1**):

(i). the molecule should have a logP value of at least 2.92 or higher, to permit hydrophobic/van der Waals interactions [15, 91];

(ii). the molecule should have a long chain (18 atoms or higher) to cover more than one P-gp unit, and strengthen the binding [15, 91];

(iii). the molecule should establish hydrogen bonds with P-gp [15, 91];

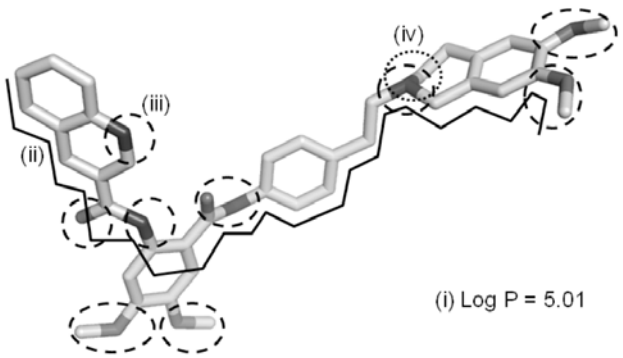


Fig. (1). Example of a third generation P-gp inhibitor (tariquidar) and the features described as being important for P-gp modulation: (i) Log P of at least 2.92, (ii) chain of more than 18 atoms (full line), (iii) sites for the establishment of hydrogen bonds (brocken line), (iv) a tertiary nitrogen atom (dotted line).

(iv). a promising P-gp modulator should have at least one tertiary nitrogen atom [15, 91], so as to form a cation at physiological pH value, and to strengthen the binding through ionic/H-bonds with P-gp. Although some compounds without a tertiary nitrogen atom also exhibit P-gp inhibitory activity, it is indispensable for highly efficient P-gp modulators to have this important structural feature [91].

(v). P-gp inhibitors would have high E_{HOMO} (highest occupied molecular orbital energy) [131].

In spite of all the molecular modeling studies performed, it would be important that each new pharmacophore or QSAR study could be compared to those previously described, so that identical or similar features or descriptors could be identified. Many types of descriptors have been applied in these studies but a general correlation is elusive. It is disappointing that to date there has been no success in establishing the conclusive structure activity relationships for inhibitory activities of P-gp interacting substances.

CONCLUSION

Although P-gp inhibitors have been known to exist for 30 years [1], the understanding of the molecular basis of interaction between those compounds and the efflux pump is still scarce, which is mainly due to the lack of structural information available. However, over the past decade X-ray structures of several bacterial homologues as well as the very Recent structure of mice P-gp have become available [6, 132]. Emerging homology models have allowed researchers to envisage how P-gp may interact with its inhibitors. In general, the results from the combined pharmacophore, docking and 3D QSAR modeling studies correspond and complement each other in revealing important structural features and could be helpful for the development of highly selective and potent P-gp inhibitors [125]. The combination of these structure and ligand-based studies with experimental datawill play a crucial role in the near future. Many of the experimental techniques like photoaffinity studies and cysteine scanning mutagenesis [133] provide important results that complement the computational results, providing useful insights into the different inhibition-binding sites of P-gp.

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COMPETING INTERESTS

The authors have declared that no competing interests exist.

ACKNOWLEDGEMENT

None declared.

ABBREVIATIONS

CADD	=	Computer-assisted drug design
MDR	=	Multidrug resistance
P-gp	=	P-glycoprotein
SAR	=	Structure-activity relationships
QSAR	=	Quantitative structure-activity relationships
3D-QSAR	=	3-Dimensional quantitative structure-activity relationships
ABC	=	ATP-binding cassette
NBD	=	Nucleotide binding domain
TMD	=	Transmembrane domain
MW	=	Molecular weight

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