Inhibitors of P-Glycoprotein – Lead Identification and Optimisation

Karin Pleban and Gerhard F. Ecker*

Department of Pharmaceutical Chemistry, University of Vienna, Althanstrasse 14, 1090 Wien, Austria

Abstract: The membrane bound drug efflux pump P-glycoprotein (P-gp) transports a wide variety of functionally and structurally diverse cytotoxic drugs out of tumour cells. Overexpression of P-glycoprotein is one of the predominant mechanisms responsible for development of multiple drug resistance in tumour therapy. Thus, inhibition of P-gp represents a promising approach for treatment of multidrug resistant tumours. This review highlights concepts for identification and optimization of new inhibitors of P-glycoprotein.

Keywords: Inhibitors of P-glycoprotein, multidrug resistance, QSAR, pharmacophore models.

INTRODUCTION

It was 1976, when the group of Victor Ling identified P-Glycoprotein (P-gp) as being responsible for reduced drug accumulation in multidrug resistant Chinese hamster ovary cells [1]. P-gp functions as a membrane bound, ATP-dependent efflux pump extruding a wide variety of functionally and structurally diverse natural product toxins out of mammalian cells [2]. Overexpression of P-gp thus leads to multiresistance to cytotoxic agents, which mainly is observed in tumour therapy. Only 5 years later Tsuruo *et al*. found verapamil to be able to block P-gp mediated transport [3]. This gives rise to a restoration of sensitivity of multidrug resistant cells to chemotherapeutic agents and thus represents a versatile approach for overcoming drug resistance. Since this, numerous compounds have been identified and several are in clinical studies up to phase III [4]. However, although much progress has been made in understanding the function and kinetics of P-gp, the mechanism of drug-protein interaction on a molecular basis is still unknown. There is no high resolution structure available yet and the multispecificity for both substrate and inhibitors renders rational drug design approaches a difficult task. Although within analogous series of compounds clear SAR-patterns exist, a general receptor model allowing virtual screening approaches is still missing. This review will focus on recent advances in generating pharmacophore models, which are more generally applicable for predicting substrate and/or inhibitor properties of structurally diverse compounds.

LEAD-IDENTIFICATION

To fasten and streamline the process of lead-identification is one of the major challenges in the drug development process. In the early 20th century, new drugs were mainly discovered via serendipity and modification of natural products. Prominent examples are chlorodiazepoxide and penicilline. In the sixties and seventies, often denoted as the golden age in drug development, transition state mimicry was the method of choice. This approach works excellent for enzymes and proteases. During this time, also molecular modeling came up and lead optimisation programs were put on a more rational basis using QSAR methods. Although big efforts were put on development of highly sophisticated computational methods, successful applications are rare and only very few examples exist where new drugs were generated solely on a computational basis. Nowadays the drug development process starts with hits obtained in HTS assays. Up to 1.000.000 compounds are biologically screened on a yes/no basis and the resulting hits are prioritised on basis of novelty, patentability, synthetic accessibility and data obtained in early ADMET profiling programs. A typical HTS library consists of both a so called historical collection (i.e. compounds from previous drug development programs) and commercially obtained substances, which are selected on basis of maximum chemical diversity combined with high drug likeliness. In parallel, *in silico* screening approaches are gaining increasing importance. They are mainly used to select subsets of large virtual combinatorial libraries, which should show a higher incidence for biological activity (or at least eliminate all non drug like compounds) and thus lead to increased hit rates. In the near future, the information from genomics and proteomics combined with bioinformatics and protein modeling will allow high quality *in silico* protein structure generation, which may then be used for high throughput docking experiments or even *de novo* design of high affinity ligands via "on the fly" enumeration of combinatorial libraries within the binding site.

In the field of P-gp inhibitors lead identification mainly was based on serendipity. First attempts of identifying a general pattern of pharmacophoric structures were performed by Klopman *et al*. The authors used the MULTICASE software package to analyse a set of structurally diverse ligands of P-gp [5]. They identified a set of pharmacophoric and pharmacophobic sub structures (Fig. **1**). However, looking on these one may realise that these substructures are present in almost all low molecular weight drugs. This is also reflected in the compounds identified as 1st generation P-gp inhibitors. Most of them are well established as drugs in different pharmacological areas. Clinical studies of some of the compounds very early revealed the major problem with first generation modulators: when using the compounds as MDR-modulators, the inherent pharmacological activity is rendered a side effect, which often was dose limiting

*Address correspondence to this author at the Department of Pharmaceutical Chemistry, University of Vienna, Althanstrasse 14, 1090 Wien, Austria; E-mail: gerhard.f.ecker@univie.ac.at

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(Table **1**) [6]. Thus, due to the fact that in case of the presence of stereogenic centers both isomers show almost identical P-gp inhibitory activity, some companies performed a chiral switch using those stereoisomers, which are less active in their original indication (second generation modulators, such as dexverapamil [7] and dexniguldipine [8]). However, both compounds were withdrawn from clinical studies due to severe side effects. The so called third generation modulators comprise chemical structures and seem to be exclusively developed for P-gp inhibition (Fig. **2**). Some compounds are in late stage clinical studies (Table **2**) [9-20] for a recent review see also [21]. While valspodar was developed on a rational basis starting from cyclosporin A, the other compounds seem to have been identified in lead identification protocols based on screening technologies.

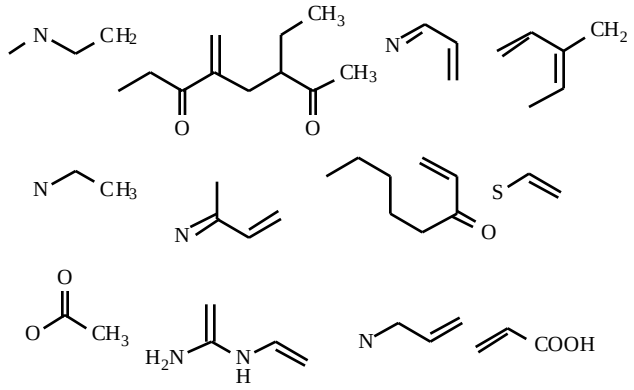


Fig. (1). Pharmacophoric substructures for P-gp inhibitors.

With respect to *in silico* screening techniques for identification of new P-gp inhibitors, we recently introduced a method based on self organizing maps (SOMs) [22]. SOMs belong to those group of artificial neural networks, which are trained on basis of unsupervised learning. They are mainly used for classification purposes and are excellently suited for identifying hidden patterns in large data sets. On principle, the objects of interest are grouped on a map according to their similarity, i.e. similar compounds are grouped in close vicinity or even are located in the same neurone. Thus, a set of descriptors is calculated for the compounds of interest and the compounds are presented to the network. After convergence is reached, the neurons with the compounds in them are color coded according to the property of interest (i.e. active = yellow, medium active = orange, inactive = red). If the descriptors were chosen right and there is a pattern in the data set, the network should have been able to separate active from inactive compounds (Fig. **3**). Subsequently, this protocol can be used to predict the properties of newly synthesised or even virtual compound libraries.

We established this methodology to identify new inhibitors of P-gp via a virtual screen of the SPECS compound library. A set of 131 propafenone-type P-gp inhibitors was taken as training set and the 2D-autocorrelation vectors were calculated for a distance up to 10 bonds. The compounds were presented to a self organising map (KMAP) and the results were analysed via color coding as outlined above (Fig. **3**). In the next step, the size of the SOM was enlarged, the propafenones were merged with the SPECS library (134.000 compounds), and the process was repeated. After the training was finished, the collisions of SPECS compounds with highly active propafenones were analysed. Seven compounds were identified, which colocalise with highly active propafenone-type P-gp inhibitors (Table **3**). Two of them showed EC_{50} values in the submicromolar range, 4 in the micromolar range and only one compound was inactive. To further validate the method, 8 compounds were identified, which colocalise with almost inactive propafenones. Only one of these compounds showed moderate activity, whereas the remaining 7 were inactive. Looking on the structures of the two highly active SPECS compounds, they are completely different from propafenone and are new in the field of P-gp inhibitors [23]. Thus, this method unequivocally led to the identification of two new lead compounds.

Table 1. Achievable *In Vivo* and Optimal *In Vitro* Concentrations of 1st Generation MDR Modulators

Compound	achievable *in vivo* conc.	optimal *in vitro* conc.
Quinidine	4.5 – 5.6 µM	3.3 – 9.9 µM
Trifluoperazine	130 ng/ml	1 – 6 µg/ml
Tamoxifen	6 µM	- 10 µM
Toremifene	10 – 15 µM	- 15 µM
Cyclosporine	2.5 – 8.5 µg/ml	6 µg/ml
Varapamil	1 – 2 µM	6 – 10 µM

CLASSIFICATION SYSTEMS

Classification systems such as linear discriminant analysis or decision trees are a versatile approach for making yes/no decisions. Several systems were thus applied for the drug like/non drug like problem. These include decision tree algorithms [24] and artificial neural networks [25, 26]. On basis of the 609 compound data set of Klopman [27], Bakken and Jurs used linear discriminant analysis to generate models, which are able to classify multidrug resistance reversing compounds as active, moderately active and inactive (three class model) [28]. Additionally, also a two class model distinguishing between active and inactive compounds was developed. Both topological descriptors alone and a combination of topological, geometric, electronic and polar surface descriptors were used as variables. Subsequent feature selection to reduce the dimensionality led to a set of 100 descriptors, out of which 60 were topological in nature. Both sets were used for model generation, which was performed using linear discriminant analysis, k-nearest neighbour analysis and radial basis function neural networks. When the separation between active and inactive classes is large enough, a set of 6 topological descriptors was sufficient to obtain 92 % correct classifications in a model based on linear discriminant analysis. Thus, this model could serve as a screening tool for identification of new MDR-modulators.

Based on a comparison of more than 100 compounds tested as P-gp substrates A. Seelig suggested that a set of well-defined structural elements is required for an interaction with P-gp [29]. The type of recognition is mainly based on formation of H-bonds, and two different patters could be defined. The recognition elements are either formed by two

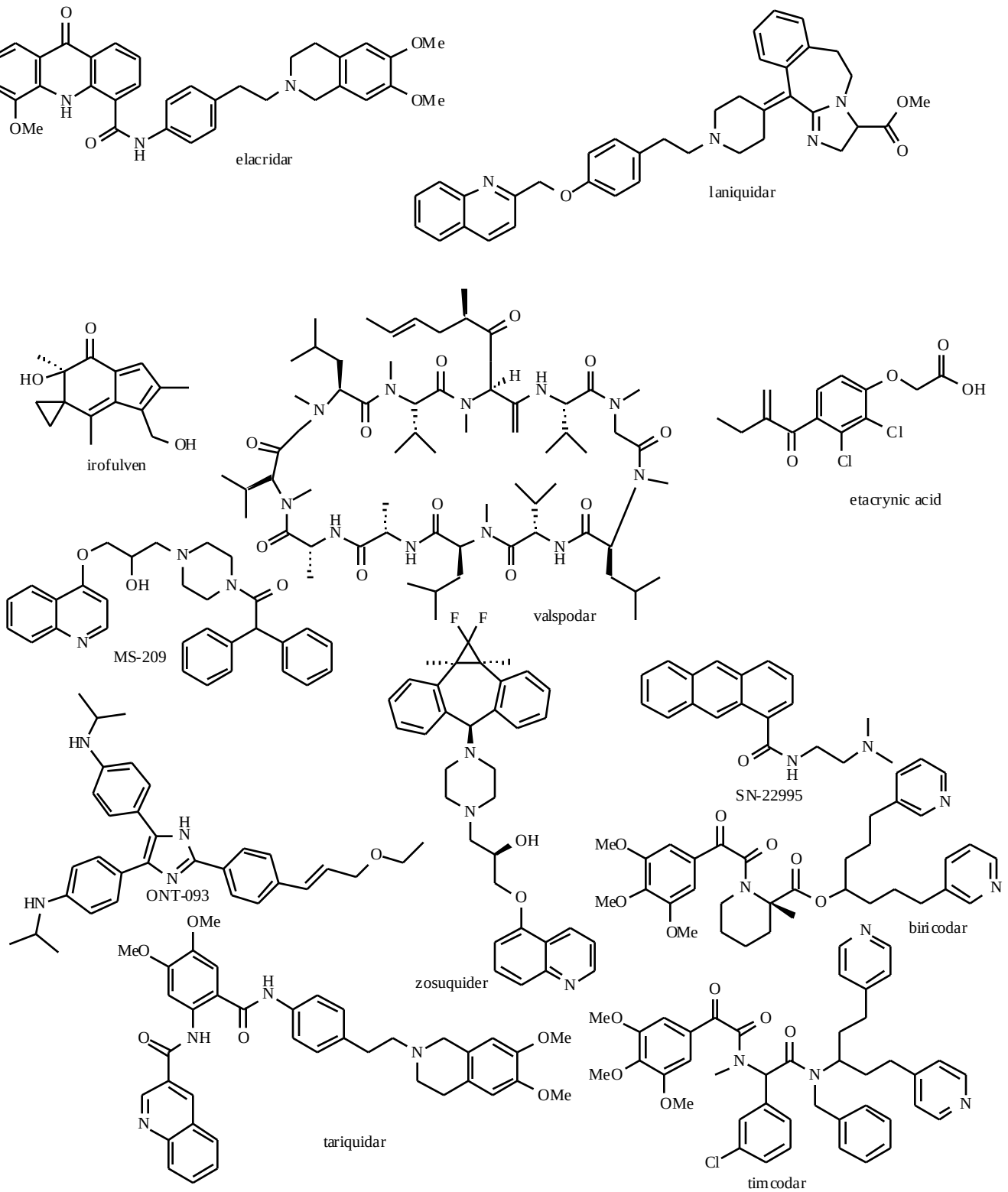


Fig. (2). Chemical structures of selected 3rd generation P-gp inhibitors.

(type I unit) or by three electron donor groups (type II unit) with a fixed spatial distance. Type I units consist of two electron donor groups with a spatial separation of 2.5 Å, whereas type II units show either 2 H-bond acceptors with a spatial distance of 4.6 Å or three electron donor groups with a distance of the outer groups of 4.6 Å. All molecules, which contain at least one type I or type II unit are predicted to be substrates of P-glycoprotein. Binding to P-gp was found to be related to the number and strength of H-bond acceptors forming the type I and II units. This hypothesis is further supported by the fact, that a high percentage of amino acids with H-bond donor sites are found in those transmembrane sequences of P-gp, which are involved in substrate interaction [30].

Table 2. Compounds Under Clinical Investigation as MDR-Modulator

Drug	Phase	Objective	Ref.
Elacridar	I	malignant neoplastic disease, solid tumours	9
SN-22995	I	solid tumours	10
Biricodar	II	prostate, lung, ovarian and breast carcinomas	11
Ethacrynic acid	II	malignant neoplastic disease, carcinoma	12
Irofulven	II	recurrent ovarian epithelial and peritoneal cancer	13
Laniquidar	II	metastatic breast cancer	14
ONT-093	II	metastatic breast cancer	15
Timcodar	II	malignant neoplastic disease	16
Zosuquidar	II	advanced solid tumours	17
MS-209	III	breast cancer, advanced solid tumours	18
Tariquidar	III	ovarian cancer	19
Valspodar	III	acute myeloid leukemia	20

A more general approach was put forward by Klopman et. al. A computer aided substructural search accompanied by a quantitative structure-activity relationship (qsar) study on 609 compounds revealed several biophores and biophobes. The most significant biophore could be expressed in the generic form C-C-X-C-C, where X = N, NH or O. Preferently the nitrogen is present in a tertiary form. Additionally, $(\log P)^2$ was identified as the most significant physicochemical term. Deactivating fragments were -COOH, phenols, anilines and quaternary ammonium compounds [27].

LEAD-OPTIMISATION

After a lead compound has been obtained, usually a lead optimisation program is started. This mainly is based on synthesis and testing of congeneric series of compounds. For inhibitors of P-gp, several systematic studies were conducted, which were extensively summarised in recent reviews [31-34]. In the following section, only those using larger sets of compounds will be addressed.

2D-QSAR Studies

Toffoli *et al*. tested a set of 14 verapamil analogues (Fig. **4**) both in doxorubicin uptake and in inhibition of photoactivated labeling of azidopine using the PGP-expressing LoVo-R human colon carcinoma cell line [35]. They showed, that no significant changes in reversion potency were obtained when a methoxy group in the phenyl ring was added (gallopamil), the methoxy groups in the phenyl ring were replaced by a Cl atom or the 1-phenyl ring was replaced by long aliphatic chains (C8-C12). A significant decrease of activity was observed for compounds with the methoxy groups replaced by H-atoms. A dramatic loss of MDR-modulating activity occurred when the cyano group was reduced to the corresponding methylamine and when the 7-cyano-8-methyl-nonane was altered by carbon extension (anipamil). Thus, both the backbone and electron donating groups on the phenyl rings seem to be necessary for high activity of verapamil derivatives.

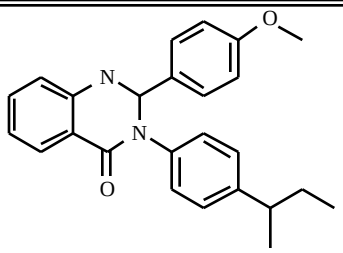
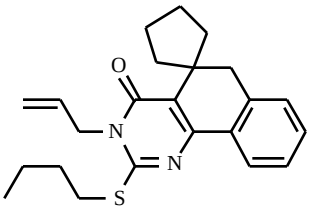
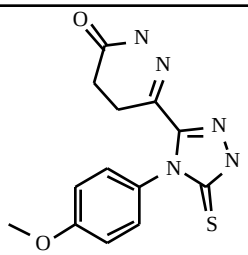
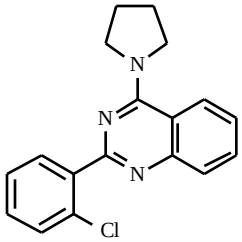
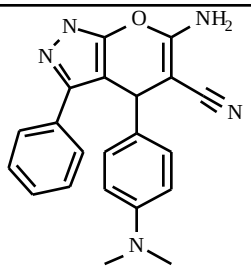
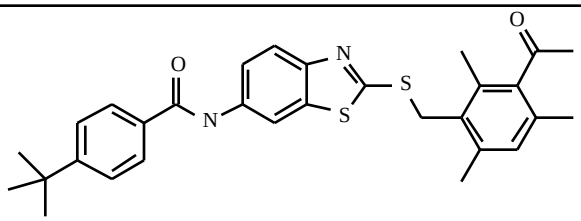
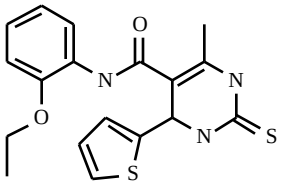
Following a systematic screening program, Dhainaut *et al*. found that almitrine moderately sensitized the multidrug resistant DC-3F/AD cell line to actinomycin D. Thus, a series of 70 triazine derivatives (Fig. **4**) was synthesized and tested for their ability to modulate multidrug resistance both in DC-3F/AD and in KB-A1 tumor cells [36]. Structure-activity relationship studies were performed on basis of systematic variations of parts A, B and C and the following results were obtained:

- For part A, a triazine ring system is beneficial to e.g. pyrimidine. Additionally, substitution with two monoalkylamino groups, preferentially allylamino, is recommended for high activity.
- The lipophilic part of the molecule (part C) should be either a benzhydryl group or a tricyclic system with a dibenzosuberane like structure. Replacing the benzhydryl group by a triphenylmethyl moiety dramatically increases toxicity with simultaneous loss of activity.
- For the spacer group between parts A and C, an amino-substituted piperidin is slightly superior to piperazine by means of both toxicity and bioavailability. Additionally, the spacer length between parts A and C is very crucial to maintain high activity. Thus, compounds with a spacer much longer then piperidylaminomethyl are almost inactive.

Due to these results, 12 compounds were selected and tested *in vivo* on sensitive P388 and vincristine-resistant P388/VCR murine leukemia. All compounds induced a significant increase in life span of the P388/VCR-bearing mice when coadministered with vincristine. The most active compound completely restored vincristine sensitivity. Nevertheless, due to favourable pharmacokinetic behaviour in dogs, S 9788 was selected for further pharmacological development. Early clinical studies demonstrated the usefulness of S 9788 in modulating resistance, but dose limiting cardiovascular side effects occurred. Moreover, calcium channel affinity of S 9788 is in the range of those from verapamil and markedly higher than that of almitrine. Further SAR-studies on this class of compounds focused on modification of the triazine moiety, whereby pyrrolopyrimidines showed highest activity. Additionally, the 4-aminopiperidine linker group was replaced by 4-methylamino-piperidine. This modification resulted in lower affinities for the calcium channel. Five analogues were also shown to completely restore sensitivity in a P388/VCR murine leukemia *in vivo* model. Measurement of the binding affinities to the phenylalkylamine binding site of the calcium channel showed, that there is no correlation between MDR-modulating activity and calcium channel binding. Thus, several compounds of the series are presently tested on cardiovascular side effects in animal models.

Dodic *et al*. synthesised and tested a set of 65 compounds originally based on amiodarone/verapamil

Table 3. Chemical Structure and Pharmacological Activity of Compounds Proposed as Highly Active in a Virtual Screen of the Specs Library

Structure	SPECS-Code	Propafenone	EC_{50} (μM)
	AG-690/11972772	GPV 610	0.76
	AG-690/12887361	GPV 576	3.50
	AJ-131/15197008	GPV 576	> 250
	AJ-292/13162028	GPV 576	5.94
	AJ-292/15089034	GPV 610	3.08
	AN-989/14669159	GPV 610	0.28
	AO-364/14480185	GPV 576	8.77

hybrides as cationic part, which is linked via an amide bond to a lipophilic tricyclic ring system (Fig. **4**). For the tricyclic system, acridone and thioxanthone was found to give best activity [37]. Replacement of acridone by 9-chloroacridine or introduction of a second carbonyl group to give an anthraquinone, decreased activity. Modification of the cationic part gave benzyl- or phenylethylamine analogues as optimum, whereby 3, 4-dimethoxy substitution was best. Rigidization of the phenylethylamine by formation of a tetrahydroisoquinoline ring further increased activity. In analoguey to the triazine derivatives, the nature and length of the spacer between the carboxamide function and the cationic nitrogen atom are also important. A simple alkyl chain (8 C-atoms) is not adequate probably due to the lack of the possibility of pi-interactions. Phenylethyl, -propyl and-butyl derivatives showed high activity. Both shorter and larger chains led to a decrease of activity. Replacement of the central phenyl ring by piperazine or piperidine led to inactive compounds. This indicates, that the carbamoylphenyl linker group contributes to MDR-activity not only via its function as spacer group, but also acts as independent pharmacophore. In light of these results, GF 120918 was tested in an *in vivo* model using mice implanted with the MDR P388/DOX tumor. Coadministration with doxorubicin gave an 50% increase of the mean survival time. Cardiovascular screening showed no calcium antagonistic activity of GF 120918, which is currently under clinical investigation.

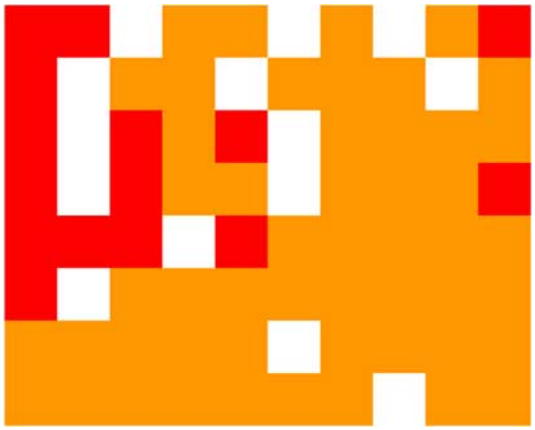


Fig. (3). Colour coded self organising map showing a separation of active and inactive propafenone-type inhibitors of P-gp; red: inactive; orange: active.

The cyclic undecapeptide cyclosporine A and its non-immunosuppressive analogue valspodar ([3'-keto-MeBmt^1, Val^2]-CsA, SDZ PSC 833) are one of the most active P-gp inhibitors [38]. This prompted Loor *et al*. to establish structure-activity relationships for a series of 60, mostly natural, cyclosporine analogues [39]. The compounds used are closely related to [Thr^2, Leu^5, D-Hiv^8, Leu^{10}]-CsA (SDZ 214-103), which is even higher active than CsA itself [40]. The SAR-data confirmed results previously obtained by use of photoaffinity labeled cyclosporines [41]. Thus, P-gp inhibition is favored by large hydrophobic side chains on cyclosporine residues 1, 4, 6 and 8. Additionally, also large hydrophobic side chains outside the putative binding region (residues 2, 3, 10 and 11) influence pharmacological activity. N-desmethylation of any of the 7 N-methylamides leads to a decrease of activity, which is highest if it occurs on residues 4 and 9, respectively. However, N-desmethyl-cyclosporins produced by *C. oligospermum* did not show this strong decrease of activity.

Pajeva and Wiese performed a series of QSAR-studies on thioxanthenes and phenothiazines (Fig. **4**) [42]. Multiple linear regression analysis showed, that in the group of thioxanthenes the length of the spacer between the aromatic ring system and the basic nitrogen atom, the substituent on the basic nitrogen atom and the stereochemistry (cis/trans) influences MDR-modulating activity. When combining this data set with a series of phenothiazines, also the type of the ring system and the substituent in position 2 significantly contribute to pharmacological activity. The results obtained especially support findings of Pearce *et al*. [43] and Suzuki *et al*. [44] concerning the role of the relative positioning of the aromatic ring system and the basic nitrogen atom. Detailed computational analysis of cis- and trans-flupenthixol combined with NMR-spectroscopic studies on the conformation of the stereoisomers in lipid environments.

One of the most intensively studied group of P-gp inhibitors are the class of propafenones (Fig. **4**). Almost 250 compounds were synthesized in our group and a series of systematic QSAR-studies were performed. For detailed results, the reader is referred to a recent review [45]. Systematic variations of the core structure in combination with various established QSAR-methods, such as Hansch-analysis, Free-Wilson analysis and the Topliss approach revealed the following pattern (Fig. **5**):

- within congeneric series of compounds (phenones, benzofuranes, benzpyranes), pharmacological activity correlates to lipophilicity and/or molar refractivity of the compounds [46-48].
- a distance of 3 – 4 methylene between the central aromatic ring and the nitrogen atom represents the optimum [49].
- altering the H-bond acceptor strength in the vicinity of the nitrogen atom influences activity independently from partial logP values [50].
- the carbonyl group acts as H-bond acceptor [51].
- substitution on the central aromatic ring influences activity mainly via its effect on the H-bond acceptor strength of the carbonyl group [52].

These 2D-QSAR studies were further extended using the new technique of Molecular Holograms, which are related to 2D fingerprints [53]. Given an input molecule, all possible structural fragments (including overlapping fragments) that contain a user-defined minimum and maximum number of atoms (4-7) are generated. A unique integer identifier is then generated for each of the resulting types of fragments and these integer identifiers are hashed into an L-integer array of a user defined length (353, 307, 257, 199, 151, 97, 83, 71, 61, 59, 53, 43, 41, 37, 31, 29, 23) to give the molecular hologram. All compounds were built in Sybyl 6.6 and minimised with the Tripos force field engine using the Gasteiger Hueckel charges. All possible combinations of informations (atoms, bonds, connections, hydrogen-bond donor-acceptor group) were used to generate the holograms. The holograms of the set of the molecules were used as input in a linear PLS analysis (maximum number of components: 10; each model was cross validated

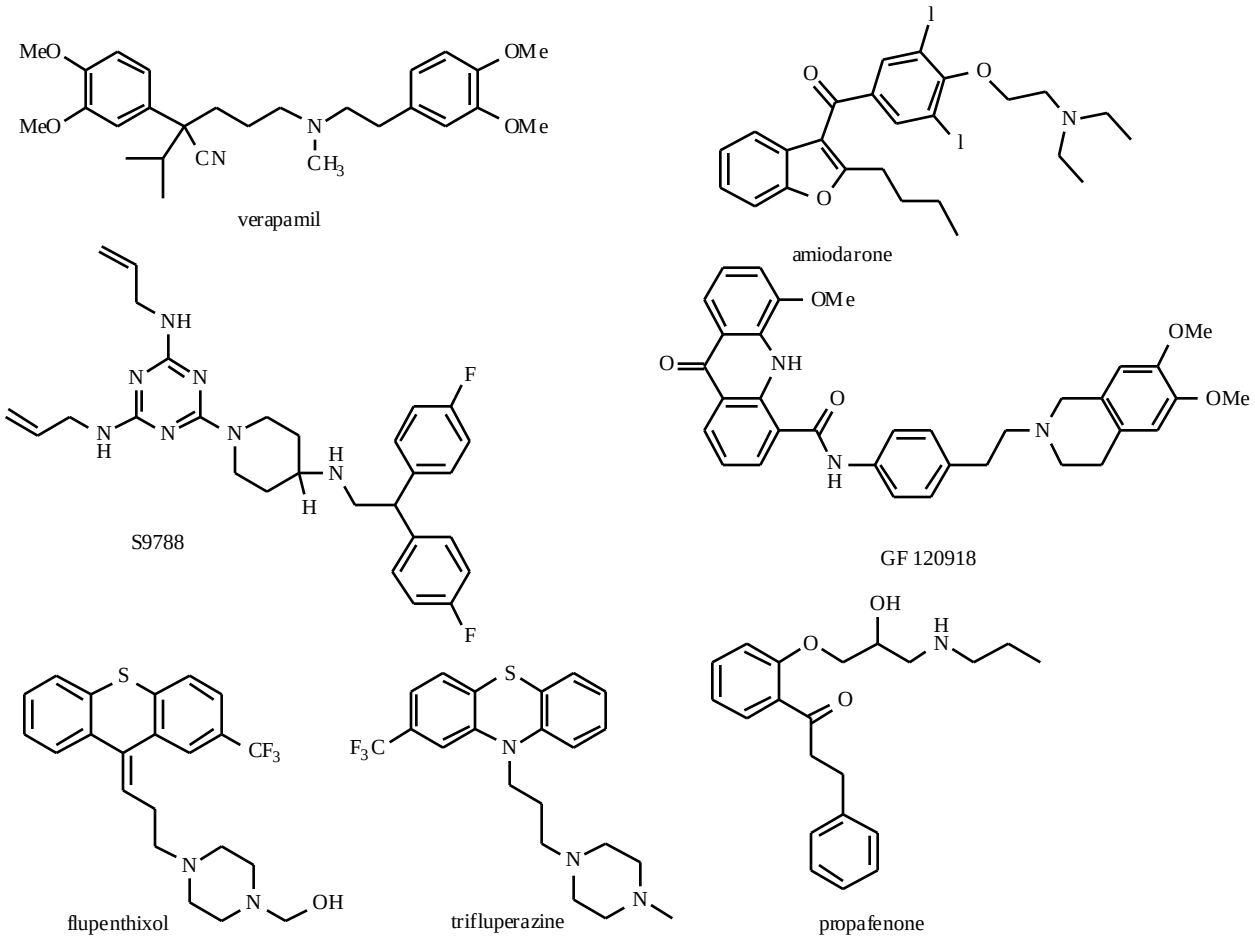


Fig. (4). Chemical structures of lead compounds used in 2D-QSAR studies of MDR-modulators.

automatically with 50 different groups). As output the pharmacological activity expressed as $\log(1/EC_{50})$ values were used.

For each model the best hologram length was used to calculate the r^2 and q^2 values. The obtained r^2 values were between 0, 717 and 0, 877 and q^2 values between 0, 564 and 0, 725.

The best model was used to analyse the influence of given substructures on PGP-inhibitory activity. This is typically done via color coded display of the respective structure diagrams.

Positive influence was found for the basic nitrogen atom, the phenyl ring (the importance of this region rises when an electron rich system like naphthyl-, p-metoxyphenyl- or a p-

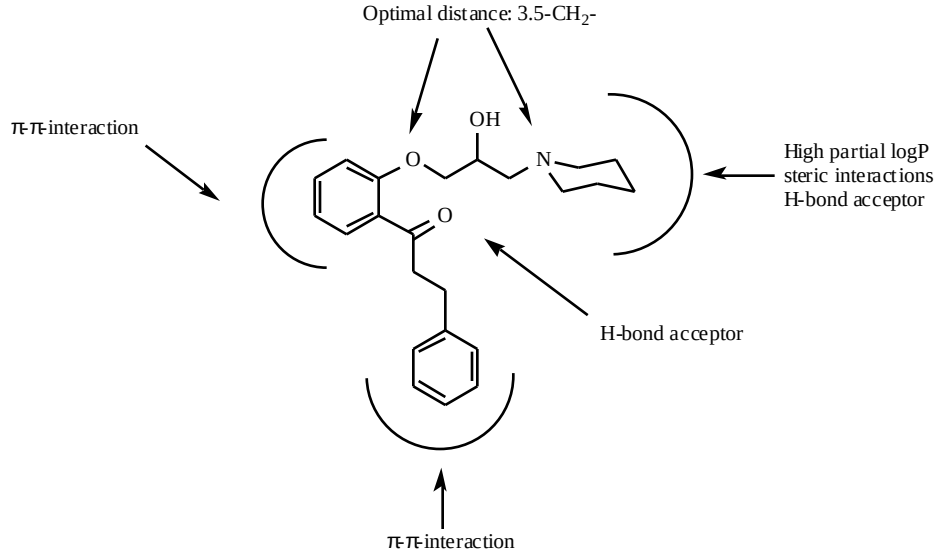


Fig. (5). Summary of the results of structure-activity relationship studies on propafenone-type inhibitors of P-gp.

dimehtylaminophenyl is present, which indicates a π-π interaction). Highly negative influence was found for diphenylalkylamines in the propanolamine chain, which is a hint for steric hindrance [54].

3D-QSAR Studies – Pharmacophore Models

In relation to the importance of the field and the numerous possible clinical applications of inhibiting human ABC-transporter (anticancer therapy, Alzheimer's disease, Tangier disease, cystic fibrosis, hyperglycemia, hypercholesteremia), only very few 3D-QSAR studies were performed up to now. Pajeva and Wiese published two CoMFA studies relying on phenothiazines, thioxanthenes [55, 56] and on a set of propafenones synthesised and tested in our lab [57, 58]. The 3D-QSAR models derived (more than 100) involved steric, electrostatic and hydrophobic fields alone and in combinations. Steric and electrostatic fields could not fully explain the variance in the data set. The best models obtained all included the hydrophobic field, which further demonstrates hydrophobicity as a property of primary importance for P-gp inhibitory activity [59, 60]. Although this was shown already in numerous studies, in case of the CoMFA studies hydrophobicity has to be considered as a space directed property rather than a descriptor for membrane distribution. Further evidence for the space directionality of lipophilicity was obtained using hydrophobic dipoles calculated on basis of interaction energies with surrounding probe atoms [61].

In an extension of these studies, we performed both a CoMFA and a CoMSIA study on a set of 131 propafenone-type P-gp inhibitors. 3D models of all tested compounds were constructed using the molecular structure builder CORINA [62] and transferred to Sybyl 6.6. In the models the asymmetric carbon atom in the propanolamine side chain was assigned R configuration as previous studies with pure isomers showed that the activity of R-isomers is slightly higher than those of the S-isomers. Dihedral angles of the models were adjusted according to similar compounds found in the CSD and afterwards the geometry was fully optimised with semiempirical AM1 method. Atomic charges were loaded from AM1 output file. Optimised models were aligned to the model of the most active compound GP576. Pharmacophoric functional groups reported in previous SAR studies were used for pair-wise RMS fitting (three aromatic ring systems represented by their centroids; carbonyl oxygen atom of the phenone side chain; nitrogen atom of the propanolamine side chain). All possible combinations of fields and logP values were correlated with $\log(1/IC_{50})$ values in PLS analyses. Cross-validated leave-one-out Q^2 values and optimal number of components N_{opt} were recorded for each run. Next, models with N_{opt} were evaluated and non-cross-validated R^2 values and standard error of estimation SEE were calculated.

The results derived from the models with highest Q^2 showed consistency with recent SAR studies [63]. Lipophilicity seems to be a very important feature for design of potent multi-drug resistance modulators, as the model where logP was included usually score higher Q^2. The most active compounds are those with high logP. An important unfavourable steric interaction was observed in compounds with too bulky substituents (e.g. diphenylmetyl) on the aminopropanol nitrogen atom. A favourable steric interaction was observed in the region of phenyl ring B, i.e. more bulky substituents should improve activity. In case of electrostatic interactions, both carbonyl oxygen and propanolamine nitrogen atoms are important for high activity. Favourable hydrophobic interaction occurs in the vicinity of phenyl ring C. The results thus support the study from Pajeva and Wiese.

Although CoMFA studies enable insights into the structural requirements on a threedimensional basis, they rely on congeneric series of compounds and are most often not expandable to sets of diverse compounds. This is mainly due to the requirement of an alignment of all molecules. Very recently Pajeva and Wiese published a general pharmacophore model of P-gp based on a highly diverse set of P-gp modulators [64]. The model is based on binding data for 25 diverse compounds to the verapamil binding site. Definition of pharmacophores and alignment was utilised using the genetic algorithm based similarity program GASP. The general pharmacophore pattern proposed involves two hydrophobic planes, three hydrogen bond acceptors (HBA) and one hydrogen bond donor (HBD). On basis of this model it is hypothesised that the binding of affinity of the compounds to the verapamil site depends on the number of the pharmacophores simultaneously involved in the interaction with P-gp. Thus, different drugs can interact with different receptor points in different binding modes, which may explain the multispecificity of P-gp.

Garrigues *et al*. determined both the binding affinities and the influence on ATPase activity for a series of compounds and used the results for molecular modeling studies [65]. Calculation of intramolecular distribution of hydrophobic and polar surfaces enabled the superposition of some compounds on basis of their surface similarity. This led to the identification of two different, but partially overlapping binding pharmacophores, which increases the possibility for multiple chemical structure recognition. One pharmacophoric site is defined by one aromatic area, two alkyl areas and electron donor (H-bond acceptor), and the other by one aromatic area, three alkyl areas and one electron donor.

Based on the study of Seelig, Penzotti *et al*. set up a series of three and four point pharmacophore models, which allowed correct classification of 60% of P-gp substrates and 80% of the non-substrates [66]. A training set of 144 compounds was used and a maximum of 100 conformers for each compound were generated. Out of three million pharmacophores sampled by these molecules, approximately 3450 had an information content greater than that expected from random noise level. Furthermore, 1030 models showed an information content level greater than one standard deviation above the mean random noise. The 100 top most informative models, denoted as pharmacophore ensemble, contains 53 four-point pharmacophores, 39 three-point pharmacophores, and 8 two-point pharmacophores. 88 out of these pharmacophores contain at least one H-bond acceptor. Both patterns for H-bond acceptors described by Seelig are represented in the models [67]. Half of the models consist of the combination H-bond acceptor, H-bond donor and hydrophobic area. This ensemble may be applied for screening of virtual libraries for P-gp substrates in the near

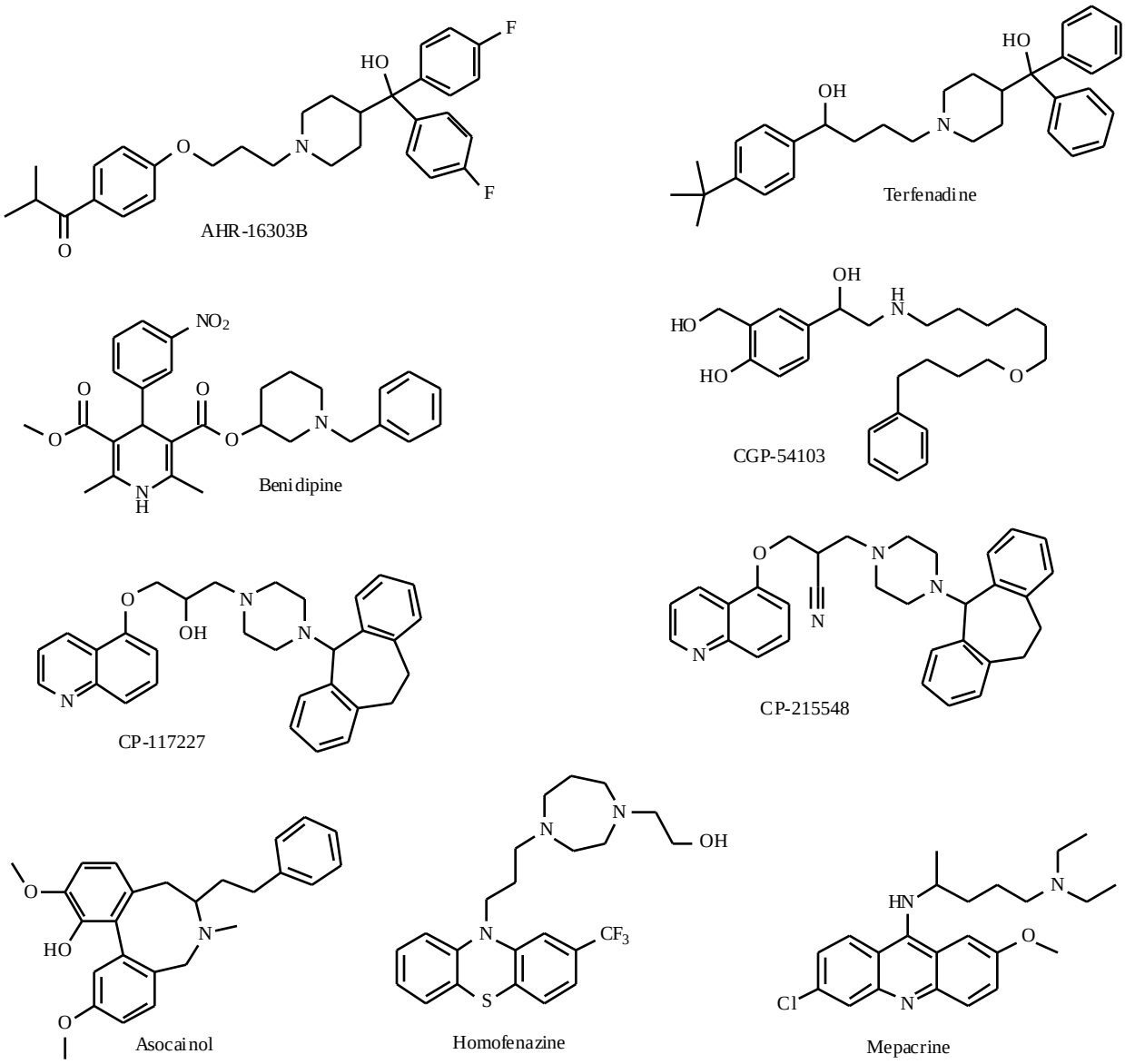


Fig. (6). chemical structure of the compounds identified in a virtual screen of the world drug index based on a 3D-pharmacophore model derived with CATALYST.

future. This might undoubtedly be of importance for early ADME profiling of compound libraries.

Ekins and coworker used the software package CATALYST to derive 3D-QSAR studies of inhibitors of P-gp. CATALYST works via a chemical function based pharmacophoric feature modeling. Thus, pharmacophoric features, such as hydrophobic, aromatic, H-bond acceptor, H-bond donor, and positively or negatively charged, are assigned to the respective sub structures of the molecules. Then, a multiconformational database is generated and QSAR models are derived linking special arrangement of pharmacophores in space to pharmacological activity of the compounds. Ekins *et al*. retrieved 4 models out of their studies [68]. One model was built on basis of 27 inhibitors of digoxin transport in Caco-2 cells and showed four hydrophobic areas and one hydrogen bond acceptor. A second model based on 21 inhibitors of vinblastine binding to plasma membrane vesicles (CEM/VBL100 cells) consisted of three aromatic ring features and one hydrophobic area. Using 17 inhibitors of vinblastine accumulation in P-gp expressing LLC-PK1 cells, a model with four hydrophobes and one H-bond acceptor was obtained. Interestingly, analysing inhibition of calcein accumulation in L-MDR-1 cells, a pharmacophore with two hydrophobic features, one H-bond acceptor and one H-bond donor was obtained. Comparing the different models on basis of their predictive power, the digoxin transport inhibition model and the vinblastine binding inhibition model showed quite equivalent results. This suggests some commonality in their binding sites. On basis of these models, inhibition of binding of verapamil to P-gp was predicted for a set of 16 compounds. Additionally, a fifth model was generated on basis of the data from the 16 compounds, which correctly ranked the 4 data sets described in the previous study [69]. This model consists of one hydrogen bond acceptor, one aromatic ring feature, and two hydrophobic areas. This further strengthens the hypothesis,

that verapamil, vinblastine and digoxin bind to overlapping sites on P-gp.

Focusing on similarity based in silico approaches for identification of new inhibitors of P-gp [70], we generated a pharmacophoric feature model on basis of 27 propafenone-type inhibitors of daunorubicin efflux from P-gp expressing CCRF-CEM vcr1000 cells. The model consists of one hydrogen bond acceptor, two aromatic regions, one hydrophobic area and one positively chargable group. This model was validated with additional 81 compounds from our in house data base and then used for screening the Word Drug Index. After adding a shape constraint to the query, 32 structurally diverse compounds were retrieved. Nine our of these are already described in the literature as MDR-modulators and comprise derivatives from different pharmacological groups, such as dihydropyridines, terfenadine analogueues, phenothiazines and chloroquine analogues (Fig. **6**) [71]. Thus it seems likely, that also the other compounds may show some P-gp inhibitory activity. This approach definitely led to the identification of new lead compounds in this field, which somewhat closes the circle to the first chapter of this article (Lead-Identification).

STRUCTURAL APPROACHES

When applying structure-based drug design approaches, high resolution X-ray or NMR-based structures are an absolute requirement. However, when dealing with membrane bound proteins, this is a quite challenging task and only very few structures with a resolution higher than 3 Å are available in the Protein Data Bank. In case of ABC-transporters, the structure of the lipid A exporter MsbA [72], the vitamin B12 importer BtuCD [73] (both from *E. coli*), and the MsbA from vibrio colerae [74] were resolved recently. These structures served as starting points for protein homology modelling approaches aiming at 3D-models of P-gp [75, 76]. Additionally, extensive structural studies of the nucleotide binding domains combined with molecular modelling and molecular dynamics simulations give first insights into functional aspects, especially the coupling of ATP-binding to TMD-movement. However, currently no information is available on the structure of the substrate and/or inhibitor binding site(s). Thus, it will be a long way to go for *in silico* docking and *de novo* design approaches, which both need high resolution structures of the respective proteins together with ligands.

CONCLUSION

Although inhibition of P-glycoprotein did – in the clinical setting - not fully fulfil the high expectations, it is a promising approach for overcoming multiple drug resistance. A lot of compounds were identified and SAR- and QSAR studies gave valuable insights into structural requirements necessary for high ligand affinity. However, detailed informations on the binding mode and the molecular basis of this quite unique "directed unspecificity" is still missing. Within the past two years, X-ray crystal structures of three ABC-transporter were published, which served as basis for two models of P-glycoprotein generated via protein homology modeling. These models may pave the way for more detailed insights into the structural biology of P-glycoprotein and thus enable the application of structure based drug design methods for identification of new P-gp inhibitors.

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REFERENCES

[1] Juliano, R.L.; Ling, V. *Biochim. Biophys. Acta*, **1976**, *455*, 152-162.
[2] Gottesman, M.M.; Pastan, I. *Annu. Rev. Biochem*., **1993**, *62*, 385-427.
[3] Tsuruo, T.; Iida, H.; Tsukagoshi, S.; Sakurai, Y. *Cancer Res*., **1981**, *41*, 1967-1972.
[4] Leonard, G.D.; Polgar, O.; Bates, S.E. *Curr. Opin. Investig. Drugs*, **2002**, *3*, 1652-1659.
[5] Klopman, G.; Srivastava, S.; Kolossvary, I.; Epand, R. F.; Ahmed, N.; Epand, R. M. *Cancer Res*., **1992**, *52*, 4121-4129.
[6] Raderer, M.; Scheithauer, W. *Cancer*, **1993**, *72*, 3553-3563.
[7] Haussermann, K.; Benz, B.; Gekeler, V.; Schumacher, K.; Eichelbaum, M. *Eur. J. Clin. Pharmacol*., **1991**, *40*, 53-59.
[8] Hofmann, J.; Gekeler, V.; Ise, W.; Noller, A.; Mitterdorfer, J.; Hofer, S.; Utz, I.; Gotwald, M.; Boer, R.; Glossmann, H.; Grunike, H.H. *Biochem. Pharmacol*., **1995**, *49*, 603-609.
[9] Lokiec, F.M.; Brain, E.G.; Faivre, S.; Armand, J.P.; Gillotin, C.; Boissaye, P.; Maety, M.; Raymond, E. *Proc. Am. Soc. Clin. Oncol*., **2003**, *22*, 153.
[10] Antonini, I.; Polucci, P.; Kelland, L.; Spinelli, S.; Pescalli, N.; Martelli, S. *J. Med. Chem.*, **2000**, *43*, 4801-4805.
[11] Seiden, M.V.; Swenerton, K.D.; Matunolis, U.; Campos, S.; Rose, P.; Batist, G.; Ette, E.; Garg, V.; Fuller, A.; Harding, M.W.; Charpentier, D. *Gynecol. Oncol*., **2002**, *86*, 302-310.
[12] *Clin. Trials Monitor*, **1995**, *4*, 27-28.
[13] Baekelandt, M. *Curr. Opin. Invest. Drugs*, **2002**, *3*, 1517-1526
[14] Petrich, S.; von Minckwitz, G.; Costa, S.D.; Loibl, S.; Palmer, P.; Bol C.; Kaufmann, M. *Proc. Am. Soc. Clin. Oncol*., **1999**, *18*, 15-18.
[15] http://www.bioportfolio.com/LeadDiscovery/PubMed-010312.htm
[16] Germann, U.A.; Shlyakhter, D.; Mason, V.S.; Ford, P.J.; Harding, M.W. *Proc. Am. Soc. Clin. Oncol*., **1996**, *37*, 335.
[17] Dantzig, A.H.; Law, L.K.; Cao, J.; Starling, J.J. *Curr. Med. Chem*., **2001**, *8*, 39-50.
[18] Nokihara, H.; Yano, S.; Nishioka, Y.; Hanibuchi, M.; Higasida, T.; Tsuruo, T.; Sone, S. Jap. *J. Cancer Res*., **2001**, *92*, 785-792.
[19] Xenova Group plc., Press Release, 2002, June 28
[20] Joly, F.; Mangioni, C.; Nicoletto, M.; Manikhas, G.M.; Walker, J.; Mietlowski, W.; Jones, G.; Wysowskyj, H.; Dugan, M. *Proc. Am. Soc. Clin. Oncol*., **2001**, *21*, 806.
[21] Gottesman, M.M.; Bates, S.E. *Nat. Rev. Cancer*, **2002**, *2*, 48-58.
[22] Kaiser, D.; Terfloth, L.; Kopp, S.; Schultz, J.; de Laet, R.; Chiba, P.; Ecker, G.F.; Gasteiger, J. *Drugs Future*, **2002**, *27* (Suppl. A), 294.
[23] Kaiser, D.; Terfloth, L.; Kopp, S.; Schultz, J.C.C.; de Laet, R.; Chiba, P.; Ecker, G.F.; Gasteiger, J. *Sci. Pharm*., **2003**, *71*, S32.
[24] Wagener, M.; van Geerestein, V.J. *J. Chem. Inf. Comput. Sci*., **2000**, *40*, 280-292.
[25] Sadowski, J.; Kubinyi, H. *J. Med. Chem*., **1998**, *41*, 3325-3329.
[26] Shah, A.V.; Walters, W.P.; Murcko, M.A. *J. Med. Chem*., **1998**, *41*, 3314-3324.
[27] Klopman, G.; Shi, L. M.; Ramu, A. *Mol. Pharmacol*., **1997**, *52*, 323-334.
[28] Bakken, G. A.; Jurs, P. C. *J. Med. Chem*., **2000**, *43*, 4534-4551.
[29] Seelig, A. *Eur. J. Biochem*., **1998**, *251*, 252-261.
[30] Seelig, A. Int. J. Clin. Pharmacol. Therapeutics, **1998**, 36, 50-54.
[31] Teodori, E.; Die, S.; Scapecchi, S.; Gualtieri, F. *Il Farmaco*, **2002**, *57*, 385-415.
[32] Wiese, M.; Pajeva, I. K. *Curr. Med. Chem*., **2001**, *8*, 685-713.
[33] Stouch, T. R.; Gudmundsson, O. *Adv. Drug Deliv. Rev*., **2002**, *54*, 315-328.
[34] Avendano, C.; Menendez, J.C. *Curr. Med. Chem*., **2002**, *9*, 159-193.

[35] Toffoli, G.; Simone, F.; Corona, G.; Cappelletto, B.; Gigante, M.; Boiocchi, M.; Raschack, M. *Biochem. Pharmacol*., **1995**, *50*, 1245-1255.
[36] Dhainaut, A.; Regnier, G.; Atassi, G.; Pierre, A.; Leonce, S.; Kraus-Berthier, L.; Prost, J. F. *J. Med. Chem*., **1992**, *35*, 2481-2496.
[37] Dodic, N.; Dumaitre, B.; Daugan, A.; Pianetti, P. *J. Med. Chem*., **1995**, *38*, 2418-2426.
[38] Gaveriaux, C.; Boesch, D.; Boelsterli, J. J.; Bollinger, P.; Eberle, M.K.; Hiestand, P.; Payne, T.; Traber, R.; Wenger, R.; Loor, F. *Br. J. Cancer*, **1989**, *60*, 867-871.
[39] Loor, F.; Tiberghien, F.; Wenandy, T.; Didier, A.; Traber, R. *J. Med. Chem*., **2002**, *45*, 4598-4612.
[40] Schwabe, K.; Steinheider, G.; Lawen, A.; Traber, R.; Hildebrandt, A. *J. Cancer Res. Clin. Oncol.*, **1995**, *121*, 407-412.
[41] Demeule, M.; Wenger, R. M.; Beliveau, R. *J. Biol. Chem*., **1997**, *272*, 6647-6652
[42] Pajeva, I.; Wiese, M. *Quant. Struct.-Act. Relat*., **1997**, *16*, 1-10.
[43] Pearce, H. L.; Winter, M. A.; Beck, W. T. *Adv. Enzyme Regul*., **1990**, *30*, 357-373.
[44] Suzuki, T.; Fukazawa, N.; San-nohe, K.; Sato, W.; Yano, O.; Tsuruo, T. *J. Med. Chem.*, **1997**, *40*, 2047-2052.
[45] Ecker, G.; Chiba, P. *Recent Res. Devel. Med. Chem*., **2001**, *1*, 121-137.
[46] Chiba, P.; Hitzler, M.; Richter, E.; Huber, M.; Tmej, C.; Ecker, G. *Quant. Struct.-Act. Rel*., **1997**, *16*, 361-366.
[47] Ecker, G.; Chiba, P.; Hitzler, M.; Schmid, D.; Visser, K.; Cordes, H.P.; Csoellei, J.; Seydel, J.K.; Schaper, K.-J. *J. Med. Chem*., **1996**, *39*, 4767-4774.
[48] Hiessboeck, R.; Wolf, C.; Richter, E.; Hitzler, M.; Chiba, P.; Kratzel, M.; Ecker, G. *J. Med. Chem*., **1999**, *42*, 1921-1926.
[49] Chiba, P.; Annibali, D.; Hitzler, M.; Richter, E.; Ecker, G. *Il Farmaco*, **1998**, *53*, 357-364.
[50] Ecker, G.; Huber, M.; Schmid, D.; Chiba, P. *Mol. Pharmacol*., **1999**, *56*, 791-796.
[51] Chiba, P.; Ecker, G.; Schmid, D.; Drach, J.; Tell, B.; Goldenberg, S.; Gekeler, V. *Mol. Pharmacol*., **1996**, *49*, 1122-1130.
[52] Tmej, C.; Richter, E.; Hitzler, M.; Chiba, P.; Ecker, G.; Fleischhacker, W. *J. Pharm. Pharmacol*., **1997**, *49* (Suppl. 4), 106.
[53] Winkler, D.A.; Burden, F.R. *Quant. Struct.-Act. Rel*., **1998**, *17*, 224-231.
[54] Kaiser, D.; Chiba, P.; Ecker, G. *Proc. of the XVIth International Symposium on Medicinal Chemistry*, Bologna, **2000.**
[55] Ford, J. M.; Prozialeck, W. C.; Hait, W. N. *Mol. Pharmacol*., **1989**, *35*, 105-115.
[56] Ford, J. M.; Bruggeman, E. P.; Pastan, I.; Gottesmann, M. M.; Hait, W. N. *Cancer Res*., **1990**, *50*, 1748-1756.
[57] Chiba, P.; Ecker, G.; Schmid, D.; Drach, J.; Tell, B.; Goldenberg, S.; Gekeler, V. *Mol. Pharmacol.*, **1996**, *49*, 1122-1130.
[58] Ecker, G.; Chiba, P.; Hitzler, M.; Schmid, D.; Visser, K.; Cordes, H. P.; Csöllei, J.; Seydel, J. K.; Schaper, K.-J. *J. Med. Chem*., **1996**, *39*, 4767-4774.
[59] Pajeva, I. K.; Wiese, M. *Quant. Struct.-Act. Relat*., **1998**, *17*, 301-312.
[60] Pajeva, I. K.; Wiese, M. *J. Med. Chem*., **1998**, *41*, 1815-1826.
[61] Pajeva, I. K. ; Wiese, M. *C. R. Acad. Bulg. Sci*., **2001**, *54*, 81-84.
[62] http://www.molecular-networks.de/software/corina/index.html
[63] Smiesko, M., Chiba, P., Ecker, G. *Proc. of the XVIth International Symposium on Medicinal Chemistry*, Bologna, **2000**.
[64] Pajeva, I. K.; Wiese, M. *J. Med. Chem.*, **2002**, *45*, 5671-5686.
[65] Garrigues, A.; Loiseau, N.; Delaforge, M.; Ferte, J.; Garrigos, M.; Andre, F.; Orlowski, S. *Mol. Pharmacol*., **2002**, *62*, 1288-1298.
[66] Penzotti, J. E.; Lamb, M., L.; Evensen, E.; Grootenhuis, P. D. J. *J. Med. Chem*., **2002**, *45*, 1737-1740.
[67] Seelig, A. *Eur. J. Biochem*., **1998**, *251*, 252-261.
[68] Ekins, S.; Kim, R. B.; Leake, B. F.; Dantzig, A. H.; Schuetz, E. G.; Lan, L.-B.; Yasuda, K.; Shepard, R. L.; Winter, M. A.; Schuetz, J. D.; Wikel, J. H.; Wrighton, S. A. *Mol. Pharmacol*., **2002**, *61*, 964-973.
[69] Ekins, S.; Kim, R. B.; Leake, B. F.; Dantzig, A. H.; Schuetz, E. G.; Lan, L.-B.; Yasuda, K.; Shepard, R. L.; Winter, M. A.; Schuetz, J. D.; Wikel, J. H.; Wrighton, S. A. *Mol. Pharmacol*., **2002**, *61*, 974-981.
[70] Klein, C.; Kaiser, D.; Kopp, S.; Chiba, P.; Ecker, G.F. *J. Comput. Aided Mol. Drug Des*., **2002**, *16*, 785-793.
[71] Rebitzer, S.; Annibali, D.; Kopp, S.; Eder, M.; Langer, T.; Chiba, P.; Ecker, G. F.; Noe, C. R. *Il Farmaco*, **2003**, *58*, 185-191.
[72] Chang, G.; Roth, C. B. *Science*, **2001**, *293*, 1793-1800.
[73] Locher, K. P.; Lee, A. T.; Rees, D. C. *Science*, **2002**, *296*, 1091-1098.
[74] Chang, G. *J. Mol. Biol*. **2003**, *330*, 419-430.
[75] Seigneuret, M.; Garnier-Suillerot, A. *J. Biol. Chem*., **2003**, *278*, 30115-30124.
[76] Stenham, D.R.; Campbell, J.D.; Sansom, M.S.; Higgins, C.F.; Kerr, I.D.; Linto, K.J. *FASEB J*., **2003**, 17, 2287-2289.