Photoaffinity Labeling of P-Glycoprotein

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Abstract: The aim of the present review is to summarize recent progress in identifying substrate binding domains of P-glycoprotein by photoaffinity labeling. Preferred substrate binding regions have been identified using a number of photoaffinity ligands, including anthracyclines, the quinazoline iodoarylazidoprazosine (IAAP), dihydropyridines, taxanes and propafenones. These studies allowed identification of protein regions, which are involved in ligand interaction.

Keywords: Multidrug resistance, P-glycoprotein, affinity labeling.

INTRODUCTION

One major obstacle in chemotherapeutic treatment of human cancers is the occurence of cross-resistance to a panel of drugs, when exposed to a single drug. This type of resistance has been termed multidrug resistance (MDR). One major mechanism for MDR is related to the expression of P-glycoprotein (P-gp) [1, 2]. P-gp mediates the ATP-dependent extrusion of a large number of structurally unrelated compounds such as taxanes, anthracyclines, epipodophyllotoxines, vinca alkaloids and colchicin. The substrate spectrum is broad, but well defined. P-gp is a member of the ABC-transporter family of proteins [3]. Members of this group of proteins are characterized by the presence of two membrane-bound domains (TMDs), each containing six transmembrane segments and two nucleotide-binding domains (NBDs). There are three isoforms of P-gp, encoded by a small multigene family, namely *mdr* class I, II and III. Only rodents express all three forms of P-gp. In humans only class I and III are found, but class III does not confer MDR.

So-called chemosensitizers (MDR reversers, modulators) are able to decrease drug efflux and increase cellular drug accumulation, hence reversing MDR *in vitro*. Highly active compounds have high lipophilicity, aromatic ring systems and most substances contain a tertiary nitrogen atom, which is positively charged at physiological pH. A correlation between lipophilicity and pharmacological effect is observed. Substituents of the nitrogen atom influence transporter activity by contributing to overall lipophilicity of the molecule and by triggering hydrogen bond acceptor strength in the vicinity of the nitrogen atom [4, 5].

Human P-gp (MDR1) is a 170 kDa integral membrane protein, located in the plasma membrane, causing the active extrusion of cytotoxic drugs from otherwise drug-sensitive cells. P-gp has 1280 amino acids organized in two homologous halves, each comprising 610 amino acids, which are separated by a cytoplasmatic linker of 60 amino acids. Each of the two halves consists of 6 TM segments and 2 NBDs and is likely to have arisen by gene duplication. At present a high-resolution crystal structure is not available and the molecular mechanism of transport is still incompletely understood.

One possibility to explore P-gp-structure and function is the usage of photoreactive substrates, which are substrate analogs, inhibitors or nucleotide analogues. Photoaffinity labeling is defined as "a technique in which a photochemically reactive molecular entity, specifically associated with a biomolecule, is photoexcited in order to covalently attach a label to the biomolecule, usually via intermediates" [6]. Hence a target protein is directly probed through a covalent bond, which is photochemically introduced. Several photoaffinity labels have been synthesized and used as photoprobes for P-gp. Photolabeling of P-gp has been subject to reviews by Beck and Qian [7] and more recently by Safa [8], Greenberger [9] and Dey *et al.* [10].

The present review attempts to summarize recent developments with respect to photoaffinity labeling of P-gp and reports latest efforts and results since 1998.

OVERVIEW

A photoaffinity label is characterized by the presence of a photoreactive group. When the label is incubated with a target protein and irradiated with ultraviolet or low wavelength visible light, the label is covalently linked to the target protein. A limited number of photoreactive groups is used in photoaffinity labeling studies. These are arylazides, diazirines, carbenes and benzophenones [11]. Azides are commonly used because they are comparatively easy to prepare. Light sensitivity and a single activation cycle are disadvantages experienced with this type of ligands. In addition, nitrenes, which represent the reactive intermediates, are a less effective labeling species than *e.g.* excited carbonyl groups. Furthermore, singlet phenylnitrenes may form ketenimines causing a decrease in labeling efficiency. Ketenimines may also migrate away from the binding pocket to label remote nucleophiles in a non-specific manner. Substitutions on the aromatic ring bearing the azido-group may improve labeling efficiency and specificity by retarding the formation of undesired ketenimines: Affinity labels such

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as 3-aryl-3-trifluoromethyldiazirines seem to be promising candidates [12]. Diazirines show excellent chemical stability prior to photolysis and they can be rapidly photolyzed at wavelengths beyond the UV absorbance region for proteins but often require tedious multi-step synthesis. Benzophenones combine a number of advantages, including stability at ambient light, the possibility of repeated activation cycles, unambiguous photochemistry and photoactivation at higher wavelength, thus keeping protein damage at a minimum. In addition, the relatively short half-life prevents diffusion of the reactive species from the binding sites.

The use of a photoligand should be based on prior quantitative structure activity relationship studies in order to assure an unchanged mode of interaction with the target protein. It must, however, be borne in mind that introducing a photoreactive group may change the structure of a ligand molecule. Secondly, even if QSAR studies indicate an unchanged mode of interaction with the target protein, sites distant from the actual drug-binding site may be labeled, since the photoactive group might be located in a part of the molecule that does not represent a pharmacophore. In contrast, if located close to a pharmacophoric substructure, the latter might be disrupted by introduction of the photoreactive moiety.

As described by Dorman and Prestwich [11], identification of drug-target interactions using photoaffinity labeling can be performed at three levels. Firstly, binding of the drug to the intact target protein is studied on a macro level. Frequently, this method is also used to assess the effect of newly identified inhibitors and to draw conclusions with respect to the kinetics and stoichiometry of the transport process or to assess the effects of allosteric modulators on the photoligand substrate transport process [10, 13-15].

A number of studies attempted to characterize the binding domain of substrate analogs in more detail. In these experiments the protein is chemically or proteolytically degraded to peptides and those peptides, which contain covalently bound ligand molecules are characterized by sequencing, immunological mapping or mass spectrometry. This allowed identification of protein regions, which are involved in ligand binding, on a semi-micro level, *i.e.* binding of the photoligands was assigned to peptide fragments of varying length. Identification of interacting amino acid residues is normally attempted in a third step in order to establish the three dimensional structure of the ligand protein complex on a micro-level. Ideally, this is achieved by X-ray crystallography or solution phase high resolution NMR spectroscopy. Membrane proteins, however, are notoriously resistant to crystallization and P-gp is too large to be structurally resolved by available spectroscopic techniques. Mass spectrometric sequencing is an alternative if crystallographic or spectroscopic data are not available.

The following section gives an overview on photoaffinity labeling of the whole protein and experiments using limited trypsin digestion, which allow studying labeling of the N and C-terminal half of P-gp. Some of these studies have been aimed at resolving kinetic aspects of the transport process. A second section focuses on recent developments in the characterization of binding domains of P-gp substrates since 1998.

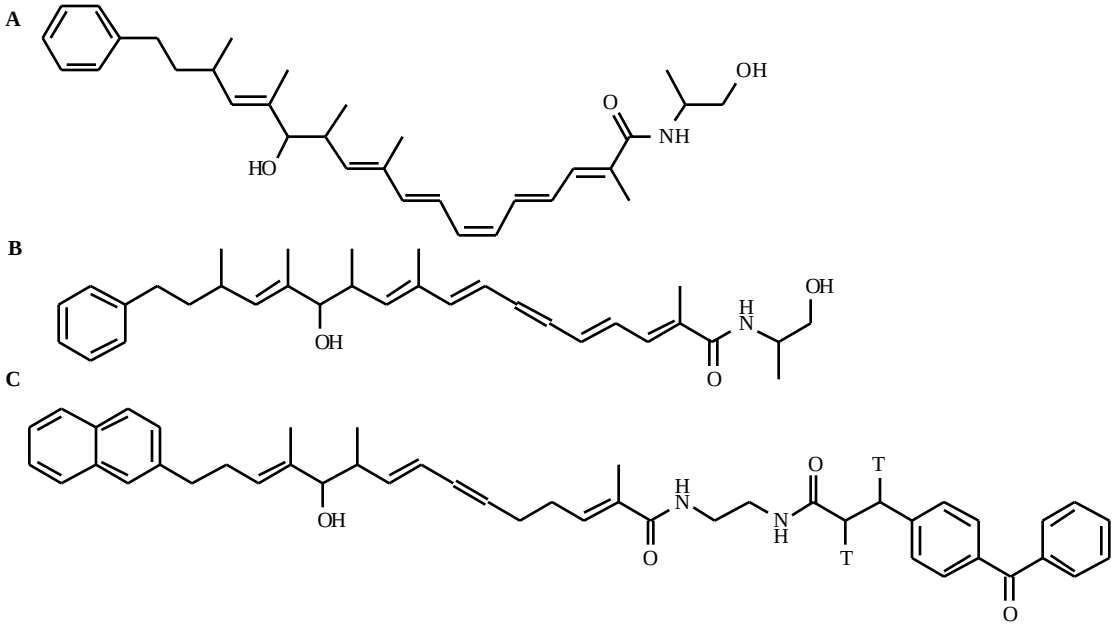
1. STUDIES ON INTACT P-GP

1.a.) Substrate and Modulator- Derived Photoaffinity Labels

Stipiamides

(-)-Stipiamide represents a highly toxic polyene natural product that acts as an MDR reversal agent. The synthetic analog 6, 7-dehydrostipiamide (DHS) (Andrus *et al.*[16, 17]) was shown to be less toxic and more potent than (-)-Stipiamide itself. A radioactive ditritiated derivative of DHS

Table 1. A.) (-)-Stipiamide B.) 6, 7-Dehydrostipiamide (DHS) C.) Radioactive Ditritiated Derivative of DHS

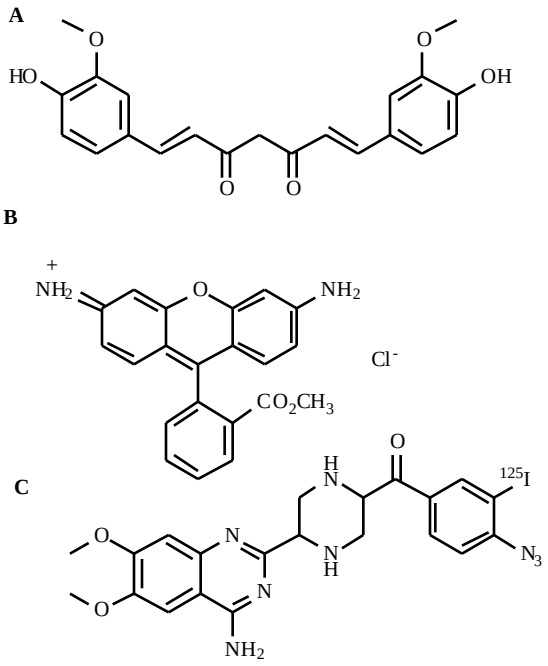


was synthesized by attaching a benzophenone moiety via a 1, 2-diaminomethane linker. This label was used as a photoligand in experiments with hMDR1-transduced High Five insect cells and hMDR1 transfected NIH3T3-cells. The P-gp band was shown to be labeled in a cyclosporine A inhibitable manner.

IAAP-Labeling Studies in the Identification of Novel P-gp Modulators

As indicated above, IAAP is widely used in labeling studies for exploring properties of substrates and modulators. Anuchapreeda *et al*. [18] investigated the mechanism of the modulator curcumin. Curcumin is a natural phenolic coloring compound, found in the rhizomes of turmeric. It shows a wide range of biological and pharmacological effects and lacks toxicity in animal models. The authors performed a series of experiments with this compound. The efflux of Rh123 decreased significantly in the presence of curcumin. In addition, cells grown in the presence of curcumin showed lower levels of MDR1 mRNA and P-gp expression, thus indicating a dual inhibitory function of this modulator. Baculovirus-transduced High Five insect cells were labeled with IAAP in the presence of curcumin. Since the labeling rate decreased with increasing curcumin concentrations, a direct interaction of curcumin with the transporter was proposed.

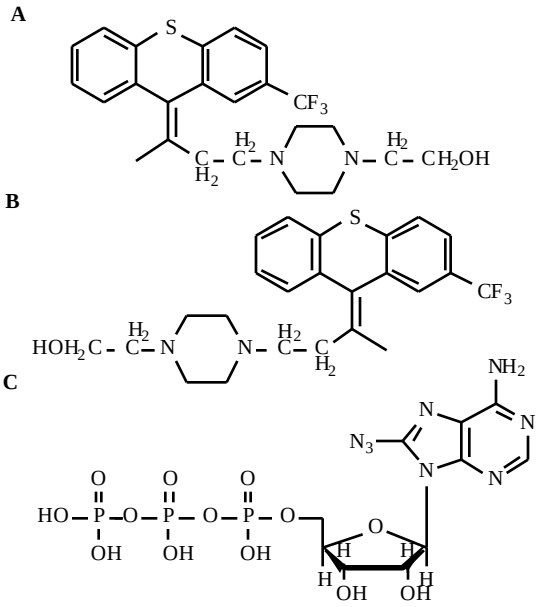
Table 2. A.) Curcumin B.) Rh123 C.) [^{125}I] IAAP



IAAP-Labeling of P-gp and Influence of Membrane Fluidity

Regev *et al*. [19] investigated the influence of membrane fluidity on P-gp function using CHO cells. ATPase activity was measured in the presence of benzyl alcohol, chloroform, ether and detergents. In the presence of benzyl alcohol both the basal and the valinomycin-stimulated ATPase activity were decreased. In photolabeling studies using [^{125}I] IAAP as the affinity ligand, benzyl alcohol was able to inhibit labeling. Anesthetics were able to accelerate the doxorubicin flip-flop rate twofold. The authors proposed that membrane fluidizers directly inhibit P-gp and that in addition net flux of the substrates is reduced by increased rediffusion rates.

Table 3. A) *Cis* (Z)-Flupentixol B) *Trans* (E)-Flupentixol C) 8-Azido-ATP

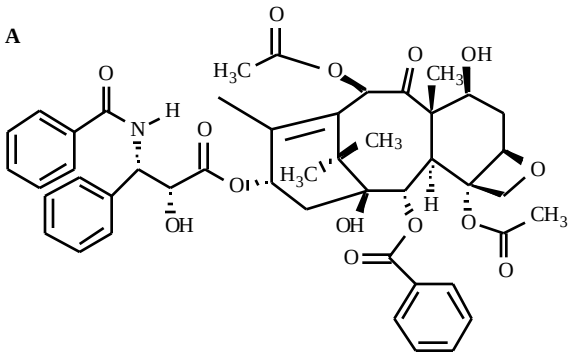


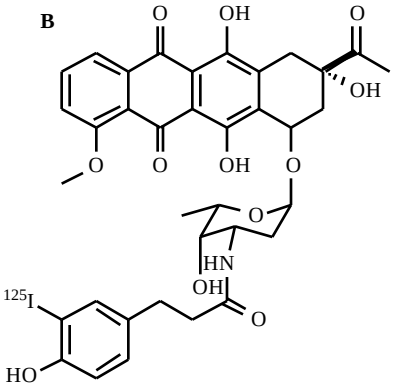
IAAP-Labeling Experiments, which Study Functional Aspect of P-gp

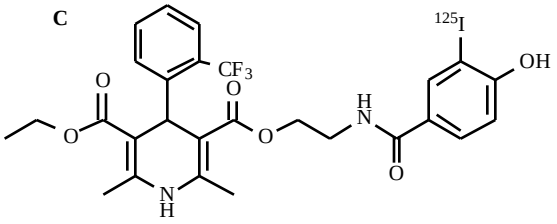
Flupentixol is an antagonist of the dopamine receptor and exists in two stereoisomeric forms, *cis*(Z)- and *trans*(E)-flupentixol. Both isoforms inhibit P-gp mediated drug efflux. While *cis*(Z)-flupentixol stimulates photolabeling with IAAP and ATP-hydrolysis, *trans*(E)-flupentixol inhibits both. Hafkemeyer *et al*. [13] created seven mutants by substitution of non-conserved amino acid residues. As confirmed by drug efflux and accumulation studies, mutant and wild type proteins behaved similarly with respect to drug transport and structural changes were therefore unlikely. Cell lines expressing wt and mutant proteins were subjected to accumulation studies using Bodipy FL-verapamil in the presence of *cis*(Z)- or *trans*(E)- flupentixol. Both flupentixol isomers were shown to loose their inhibitory function in the F983A mutant. The large increase in labeling with [^{125}I]IAAP in the presence of *cis*(Z)-flupentixol was lost in the F983A mutant. At the same time, labeling with IAAP became resistant to inhibition by *trans*(E)-flupentixol. A general loss of sensitivity to inhibitors was excluded, because labeling of F983A with IAAP stayed sensitive to Cyclosporin A inhibition. The following model was developed: P-gp contains two non-identical IAAP-binding sites, namely (1) an initial recognition site and (2) an exit site. Both isomers of flupentixol modulate by binding to a common site, of which residue 983 is an important part. *Cis*(Z)-flupentixol stimulates the initial binding of the substrate and prevents its translocation into the exit site, while *trans*(E)-flupentixol affects the initial recognition of the substrate, thus preventing binding and effluxing.

A recent publication [14] extends those findings and shows that *cis*(Z)-flupentixol exerts its effects via an allosteric mechanism, by preventing IAAP translocation and dissociation, resulting in a stable but reversible P-gp

Table 4. A) Taxol B) Iodomycin C) Iodipine







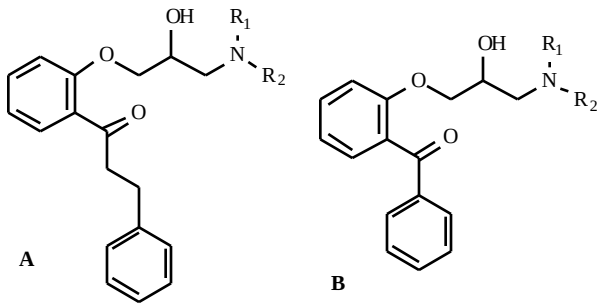
substrate complex. This allosteric modulation involves a conformational change, which is detected by the conformation sensitive monoclonal antibody UIC2.

ATP-Substrate Stoichiometry

Sauna and Ambudkar [15] investigated the catalytic cycle of P-gp with emphasis on ATP consumption. High Five insect cells transduced with a baculovirus bearing the hMDR1 insert were chosen for these studies. In P-gp ATP hydrolysis at the NBDs occurs alternately and both NBDs are required for P-gp function. P-gp can be trapped in the transition state by the addition of vanadate, which serves as a phosphate analog, but prevents posthydrolytic dissociation of ADP from the binding site. Trapping of one site arrests hydrolysis in both NBDs. ATP as well as its affinity analog 8-Azido-ATP trapped P-gp in the transition state in the presence of vanadate. Trapping abolished subsequent photolabeling with [^{125}I]IAAP. *Cis*(Z)-flupentixol was unable to stimulate IAAP. The amount of incorporated 8-Azido[α-^{32}P]ADP decreased over time and a concomitant increase in IAAP labeling was observed. Release of 8AzidoADP required an additional round of ATP-hydrolysis. After regeneration of P-gp in ATP-containing medium, a conformation similar to that observed prior to trapping was reached: IAAP binding was regained and could be stimulated by flupentixol and inhibited by Cyclosporin A.

The following transport model was developed: A drug binds to P-gp at the high affinity on-site accompanied by a concomitant binding of ATP. Hydrolysis of ATP converts the high affinity on-site into a low affinity conformation. The substrate leaves the on-site entering the off-site. The drug is extruded and the transporter is regenerated by hydrolysis of an additional molecule of ATP. Hence Sauna and Ambudkar suggested a ratio of ATP:substrate of 2:1, where the hydrolysis of an additional molecule of ATP was proposed to be essential for the catalytic cycle to advance to completion.

Table 5. A.) Propafenone Analogs B.) Benzophenone Analogs



IAAP Labeling of P-gp Mutants

Song *et al.* [20] introduced mutations both on the hydrophilic and the hydrophobic face of the α-helical TM6-segment of hamster P-gp1. The mutants were screened and EC_{50} values for different substrates were determined. Substitutions on the hydrophilic side of the helix disrupted P-gp1 function while replacement of hydrophobic residues led to functional changes. The mutants were also used in photolabeling studies with the affinity label IAAP. In

A339G, F332A, S341Q, A334F labeling only occurred when the amount of protein was increased tenfold. Hence only the affinity for IAAP was concluded to be reduced and mutations seemed to interfere with the drug interaction, when located on the hydrophilic face of the helix.

1.b.) Nucleotide-Based Photoaffinity Labels

Characterization of N-Glycosylation Mutants as a Prerequisite for Protein Crystallization

Human P-gp has three N-glycosylation sites (Asn-X-Ser/Thr) localized in the first extracellular loop at positions 91, 94 and 99. Due to glycosylation P-gp isolated from mammalian cells migrates at 170 kDa. Urbatsch [21] developed an expression system for human MDR1 P-gp in *Pichia pastoris*, which was intended to serve as a high yield expression system for subsequent crystallization. P-gp expressed in this methylotrophic yeast strain migrated with an apparent molecular mass of 140 kDa. Two mutants, in which the three Asn-residues were replaced by either Gln or Ala, were created. These two mutants were shown to have unaltered catalytic properties and similar K_m for ATP when compared to the wild type. Photoaffinity labeling was performed using 8-[α-^{32}P]azido-ATP, a good substrate for hydrolysis and photolabeling. No significant differences between labeling of wt MDR1 and the mutants could be detected. Trapping of the protein in the posthydrolytic transition-state conformation was also unaltered in the glycosylation deficient mutants. The lack of N-glycosylation in wt was confirmed by MALDI-TOF-mass spectrometry.

2. STUDIES AIMED AT THE IDENTIFICATION OF SUBSTRATE-BINDING SITES

2.1.) Substrate and modulator- derived photoaffinity labels

Identification of Taxol Binding Regions by Chemical Degradation and Immunological Mapping

Taxol (paclitaxel) is a naturally occurring diterpenoid, which is found in the bark of the Pacific Yew Tree. Paclitaxel and its semisynthetic analog Taxotère (docetaxel) are members of a class of microtubule stabilizing agents, which lead to cell cycle arrest and subsequently to apoptotic cell death. Benzophenone-analogous tritium-labeled photoreactive paclitaxel analogs bearing a 4-benzoyldihydrocinnamoyl (BzDC) moiety were created and used for photolabeling studies with murine P-gp [22, 23]. Binding of paclitaxel was assigned to the C-terminal half of the protein by CNBr degradation and subsequent immunoprecipitation with region specfic polyclonal antibodies. [^{3}H]-7-BzDC-paclitaxel labeled a peptide fragment comprising TM7, the loop between TM7 and 8 and the first half of TM8, while [^{3}H]-3'N-BzDC-paclitaxel labeled a peptide fragment comprising the last 15 residues of TM12 and the subsequent region of NBD2 extending up to amino acid 1088 (Fig. **1**), taxane binding regions highlighted in red).

Geney *et al.* [24, 25] created a hypothetical paclitaxel analog, Bis-BzDC-paclitaxel, bearing two BzDC groups at the C-7 and C-3'N positions. Both benzophenone groups were replaced by 1, 1-diphenylethanol moieties as found in the post-labeling tertiary alcohol adduct. Based on the sequence conservation between murine P-gp and MsbA, the lipid A flippase of *E. coli* [26], (36% for the aminoterminal half and 32% for the carboxyterminal half), the structure of MsbA was used for subsequent modelling studies. The 3D-structure of MsbA was imported into the software package InsightII (Accelrys, San Diego, CA, USA) and the virtual photoaffinity probe was superimposed. Segments of MsbA corresponding to the labeled sections of P-gp were identified from alignments and localized onto the 3D structure of MsbA. The major part of the two labeled segments was found to be located within the intramolecular distance between the two BzDC groups. Based on these *in silico* results the presence of a unique binding region for Paclitaxel in the C-terminal half of murine P-gp was postulated.

Identification of the IAAP-Binding Sites by Proteolytic Digestion and Immunological Mapping

Greenberger *et al.* [9] (and references contained therein) used a combination of photoaffinity labeling and high-resolution immunological mapping: Membrane vesicles of P-gp-overexpressing cells were labeled with [^{125}I]iodoarylazidoprazosin (IAAP). Following degradation with trypsin, the fragments were subjected to immunoprecipitation. A set of antibodies directed towards epitopes, distributed evenly over the linear sequence of P-gp, was used. Thereby, regions comprising putative TM5 and TM6 and a region extending from the C-terminus of TM11 to the Walker A region of the second nucleotide binding domain were identified as putative binding sites for IAAP (Fig. **1**), labeled regions highlighted in green). The authors proposed the existence of partly overlapping but distinct substrate binding sites, but did not rule out the existence of additional sites which might contribute to the IAAP binding domain.

Identification of the IAAP, Iodomycin and Iodipine Binding Sites by Chemical Degradation, Gel Electrophoretic Separation of Peptide Fragments and Edman Sequencing

Demmer *et al*. [27] used [^{125}I]iodomycin, an anthracycline, or [^{125}I]iodipine, a dihydropyridine analog, for labeling of hamster P-gp. The protein was chemically degraded with 2-(2'nitrophenylsulfenyl)-3-methyl-3'bromoindolenine (BNPS-skatol), which cleaves the protein carboxyterminally of tryptophane residues. Since these residues are rare (only 11 tryptophane residues are present in P-gp), the resulting cleavage products are comparatively large. This allowed separation by discontinous Tricine/PAGE. The labeling pattern was similar for iodipine and iodomycin. For both ligands the dominant radiolabeled band migrated with an apparent molecular mass between 8 and 10 kDa. Thus the authors proposed that the same peptide was labeled by either of the ligands. After electroelution the 8-10 kDa band was further purified using RP-HPLC on a phenyl column and subsequently subjected to 9 cycles of automated Edman sequencing. Iodipine and Iodomycin were concluded to bind to a common domain in hamster P-gp1, spanning amino acids 230 (in TM4) to 312 (in TM5) (Fig. **1**), binding sites highlighted in black).

The same group (Isenberg *et al.* [28]) continued these experiments using IAAP as the affinity ligand. Labeled protein was digested with the endopeptidase LysC. BNPS-Skatol and LysC fragments were purified as above and

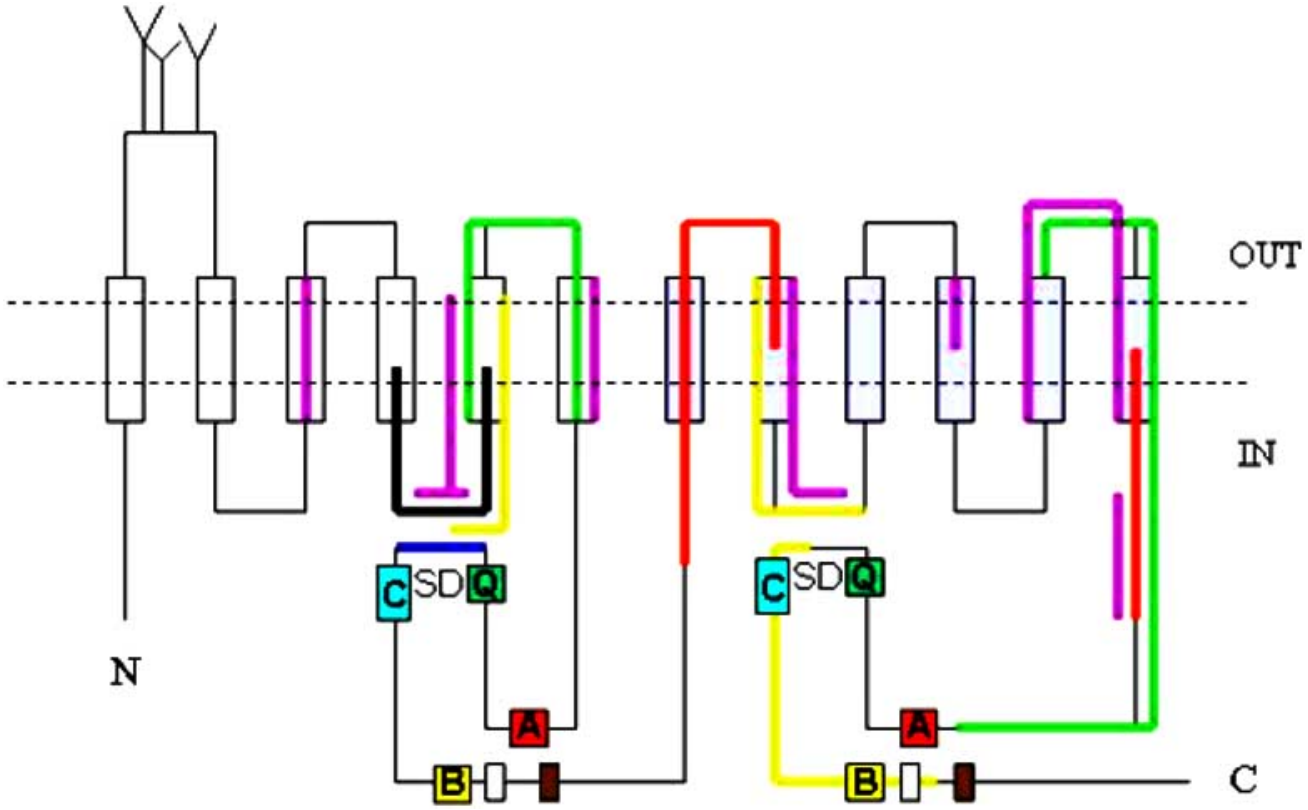


Fig. (1). Schematic representation of P-gp. Open boxes: TM-segments of N-terminal half of P-gp. Grey boxes: TM-segments of C-terminal half of P-gp. The stippled line indicates the plasma membrane. Boxes within the NBDs refer to: A: Walker A motifs (red), B: Walker B (yellow), C: signature sequence (cyan), Q: Q-loop (green). N and C refer to the amino- and carboxy terminus of the primary sequence. White boxes in the NBDs refer to the D-loop and the brown boxes to the switch region. Labeled regions for iodomycin and iodipine [27, 29] are indicated in black, those for dexniguldipine [34] in blue. Taxane binding sites are in red [22, 23], those for IAAP [28] in yellow. IAAP/azidopine/AIIP-forskolin binding regions reported in earlier publications [9] are marked in green. Magenta lines indicate consensus binding regions for propafenone analogs [39]. SD: signalling domain; the corresponding contact sequence in CL2 and CL3 shows the putative location of the sequence corresponding to the EAA-like motif of ATP-dependent importers. Glycosylation sites in ECL1 are schematically represented.

analysed by Edman sequencing. Using BNPS-Skatol degradation of the protein, a peptide fragment of 9 kDa was observed, matching the dominant band observed after degradation of iodipine- and iodomycin-labeled P-gp. Two further dominant radiolabeled bands appeared at 12 and 16 kDa. The corresponding fragments were not accessible to Edman sequencing, but became accessible after LysC digestion. By comparison and superposition of the sequencing results, three IAAP binding sites were identified. These were localized to amino acids 248-312 (corresponding to the iodipine and iodomycine binding site), amino acids 758-800 (spanning TM8 and the subsequent carboxyterminal amino acids of CL3) and amino acids 1160-1218 of NBD2 (highlighted in yellow in (Fig. **1**). Most notably, these regions comprised the sequence corresponding to the EAA-like motif of ATP-dependent importers in the N-terminal half of P-gp [29-31] and a region beyond TM8, which aligns with the L-loop of BtuC, the vitamin B12 transporter of *E. coli* [32]. Both regions have been implicated in forming an interface between TMD and NBD of ABC-transporters [33].

Identification of the Dexniguldipine Binding Site

Dexniguldipine, an enantiomerically pure dihydropyridine analog, has been designed as a P-gp inhibitor that lacks the cardiac activity associated with racemic niguldipine. The derivative [^{3}H]B9209-005 was used as a photolabel for P-gp [34]. Membrane preparations of CCRF-ADR5000, cells which overexpress human P-gp, were used in these studies. Following solubilization with N-lauroylsarcosine, the protein was separated by lectin chromatography. Western Blot analysis showed that intact P-gp (170 kDa) and an N-terminal proteolytic fragment (F1) were retrieved. The eluted proteins were subjected to digestion with varying concentrations of trypsin. Digests were separated by SDS-PAGE, gels were sliced and radioactivity was determined by liquid scintillation counting. In the absence of trypsin only P-gp and its fragment F1 were detectable. Using increased concentrations of trypsin, a shift from higher to lower molecular masses occurred. Further purification on a RP-HPLC column in the presence of N-octylglucoside led to the isolation of a 7-8 kDa fragment with a shoulder at 5 kDa. Subsequently, samples were subjected to MALDI-TOF mass spectrometry. Data recording was in linear mode. Three peaks with masses of m/z 5230, 5734 and 7416 were identified. Modification with PTC led the authors to conclude that the ion at m/z 7416 would contain at least four lysine residues and thus assigned this peptide fragment to amino acids 468-527. The authors proposed a single dexniguldipine binding site

localized within a peptide fragment extending from amino acids residue 468 to 527, thus localizing to that part of the nucleotide binding domain of the N-terminal half of P-gp which is flanked by the Walker A and B motifs. The mass peak at 5734 was interpreted to represent an unmodified peptide fragment corresponding to AA 620-670. The peak at 5230 could not be assigned. The dexniguldipine binding site is highlighted in blue in (Fig. **1**). A closer look shows that it is located between Q-loop and C-sequence of the NBD. This region is part of the helical [35] or signalling/regulatory domain [36] in prokaryotic ABC-transporters. Though the authors did not comment on the localization and a possible functional significance, this region has been implicated as an interface region between TMD and NBD of prokaryotic ABC-transporters, which reacts with the sequence corresponding to the EAA-like motif of ATP-dependent importers (or a corresponding sequence) in the TMD, thus linking ATP-hydrolysis to substrate translocation.

The lack of evidence for photolabeling of TM-segments by the dihydropyridine affinity ligand is surprising, since the TMD has been shown to confer substrate specificity for a number of ABC-transporters including P-gp. Possibly, peptide fragments from TM segments, which are highly apolar in nature, were not retrieved in RP-HPLC.

Labeling of P-gp with Benzophenone-Type Analogs of Propafenone

We previously designed and synthesized ligands, which are related to the lead compound propafenone [37]. These propafenone analogs (PAs) have substrate properties and some act as potent inhibitors of P-glycoprotein with EC_{50} values in the nanomolar range [38]. The propafenone core structure is intrinsically photoactivatable in the arylcarbonyl moiety. Compounds modified in the phenylpropiophenone substructure yield benzophenone type analogs, which show an unchanged QSAR, but are more readily photoactivated when compared to the parental compounds. Upon irradiation at wavelengths above 340nm, the carbonyl group is activated to form a biradical intermediate which reacts with otherwise unreactive C-H bonds in the target protein. Molecules are activated in a substructure, which has been documented to be a pharmacophore [4]. Use of more than one photoligand favored detection of peptide fragments localizing to regions in which a specific ligand-protein interaction takes place, while disfavoring regions of unspecific labeling. Samples were irradiated in the presence of ligand, the protein was separated on a 7.5% polyacrylamide gel, the P-gp band was excised and the protein was subjected to in-gel proteolytic digestion with either trypsin or chymotrypsin. Peptide fragments were subsequently analysed by high resolution MALDI-TOF MS [39]. In trypsin digests a sequence-coverage of approximately 50% was achieved. Peptide fragments localized to protein regions in predicted extracytoplasmic and cytoplasmic loops of the TMDs and to regions within the NBDs. However, none of the putative TM-segments was recovered, since in P-gp trypsin cleavage sites (K, R) are exclusively located outside predicted TM-segments. Thus these cleavage sites are located at a minimum distance of 20AA residues. The probability of detection of peptide fragments by high-resolution MALDI-TOF-MS, however, decreases dramatically beyond a length of 16AA (unpublished results). Therefore, chymotrypsin was used in subsequent experiments. Chymotrypsin recognition sites are frequent in TM-segments. Since some TM domains contain clusters of recognition sites, a complete digest would have led to loss of smaller peptide fragments with masses below 500 Da, since these are obscured by the MALDI-matrix. We therefore used shorter digest times, which resulted in incomplete digests and a sequence-coverage of 96% for TM-segments and an overall sequence coverage of 93%. Putative TM segments 3, 5, 6, 8, 10, 11 and 12 were identified to contribute to the binding domain of propafenone-type analogs. In addition regions outside predicted TM-segments were also labeled by the ligands. AS for IAAP, iodomycin and iodipine, these included the EAA-like motif in CL2 [30, 31] and the region located carboxyterminal of TM8 which aligns with the L-loop of BtuC [32]. A region spanning AA 1018-1037 in NBD2 as well as the loop between TM11 and 12 [39] have also been found to be accessible to the photoligands (Fig. **1**), binding regions of PAs highlighted in magenta).

CONCLUSIONS

The refinement of ABC transporter structures has made considerable progress. Two crystal structures, those of MsbA, the lipid A flippase of *E. coli* [26], and of BtuCD, the vitamine B12 transporter of *E. coli* [32], have recently been published. For other ABC-transporters partial structures of the nucleotide binding domains have been obtained by X-ray crystallography, namely of HisP [40] (ABC subunit of *E.coli* histidin permease), MalK (ABC subunit of the trehalose/maltose ABC transporter in *Thermococcus litoralis*) [41] and of the ATP binding cassettes of Rad50 [42], MJ1267 [43], MJ0796 [44], human TAP1 [45] and HlyB [46] (the hemolysin B transporter of *E.coli*). Cryo-electron microscopy in combination with image analysis has been used to obtain a low-resolution structure of P-glycoprotein [47]. A high-resolution structure of P-gp, however, still remains elusive. The latter would lay a foundation for both an understanding of the molecular basis of the transport process and of inhibitor binding. Information obtained by substrate-photoaffinity labeling approaches has led to further characterization of the transport process and to identification of substrate binding domains of P-gp and allowed to link structural information to functional aspects of drug transport. Though specific amino acid residues, which are responsible for drug interaction have not been identified yet, the following general characteristics are evident from photoaffinity labeling studies (Fig. **1**):

1. The TMDs comprise the majority of the identified labeling sites.
2. TM-segments 5, 8 and 12 as well as the region beyond TM12 contribute to binding of a number of affinity ligands including iodomycin, iodipine, azidopine, IAAP, AIIP-forskolin, propafenones and taxanes, though not all substrates have been demonstrated to bind to all of these regions.
3. IAAP and propafenones label both the sequence corresponding to the EAA-like motif of ATP-dependent importers in CL2 and the region in CL3, which aligns with the L-loop of BtuC while iodomycin and iodipine were shown to label this motif.

The predominant labeling in the TMDs reflects the importance of these domains for substrate recognition. This is in agreement with data from Loo and Clarke, who showed that the TMDs of P-gp alone suffice for substrate binding [48]. The flexible nature of the substrate-binding domain, which represents the basis for its multiple-substrate-recognition of P-gp, might provide an explanation for the identification of a number of partly overlapping binding regions. This is also supported by site directed mutagenesis studies, which demonstrated that a large number of mutations in TM-segments lead to changes with respect to substrate and modulator specificity. A recent publication by Loo and Clarke shows that dependent on the nature of the substrate different conformational changes in the TM-segments of P-gp occur [49]. The authors suggest that despite this substrate induced repacking common residues might be involved in binding of different compounds. Therefore, the use of a combined approach of photoaffinity labeling and high resolution mass spectrometry together with site directed mutagenesis and homology-based protein design holds promise in contributing to an understanding of the way in which ABC-proteins transport drugs and in elucidating the mode of action of P-gp inhibitors.

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ABBREVIATIONS

MDR	=	Multidrug resistance
ABC-transporter	=	ATP-binding cassette transporter
TMD	=	Transmembrane domain
NBD	=	Nucleotide binding domain
PA	=	Propafenone analog
MALDI-TOF-MS	=	Matrix-assisted-laser-desorption/ionization time of flight mass spectrometry

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