Anthony M. George *Editor*

ABC Transporters —40 Years on

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Foreword

Forty years after their discovery ATP-binding cassette (ABC) transporter proteins continue to fascinate and attract new generations of investigators. At its heart is the recognition that the ability of living organisms to regulate the trafficking of molecules across biological membranes is necessary for sustaining life, and that all life forms (from bacteria, to plants, to humans) have devoted a significant proportion of their genomes to evolve superfamilies of ABC transporters to meet this critical need. What are the normal physiological functions of these transporters? How do they work? What are the "rules" that govern the kind of molecules transported? Are they related to known human diseases? Can they be exploited for drug development? These are some of the key questions being investigated.

Originally, the problem of multidrug resistance (MDR) in cancer chemotherapy led to the discovery of P-glycoprotein (P-gp) (ABCB1), the first ABC transporter protein identified in mammalian cells. P-gp has the remarkable capability of mediating resistance to multiple structurally diverse drugs. The clinical importance of MDR, the promise that the blocking of P-gp function could potentially reverse MDR and benefit a large number of cancer patients drove the research. The "gold rush" was on. For a historical account of its discovery and the more important milestones in the P-gp field see Gottesman and Ling (*FEBS Lett.* 2006, 580:998–1009). Key labs involved in the early days included those in Toronto (Ling, Juliano, and Riordan), Montreal (Gros), Bethesda (Gottesman, Pastan), Amsterdam (Borst) and Tokyo (Tsuruo). The term "ABC transporter" was coined and the importance of this field eloquently socialized by Chris Higgins who noted that the fundamental building blocks of a multi-spanning membrane domain in close partnership with an ATP-binding domain was ubiquitous in nature and used by all living systems for a wide variety of biological processes.

A second "gold rush" was triggered when Susan Cole and colleagues made the breakthrough discovery in 1992 (*Science 1992*, 258:1650–1654) that P-gp was not alone in being able to cause MDR and that another ABC transporter MRP1 (ABCB1) was also associated with an MDR phenotype, albeit with a somewhat different pattern of drug resistance. That discovery along with the earlier discovery

in 1989 that the cystic fibrosis associated protein, CFTR (ABCC7) (*Science 1989*, 245:1066-1073) also was an ABC protein opened the floodgate. Application of cloning by homology technology greatly facilitated the identification of new ABC proteins. When the dust settled, 48 ABC transporters distributed in 7 ABC families (A, B, C, D, E, F, and G) were identified in humans. The multidrug resistance phenotype relevant to cancer chemotherapy had arisen independently in at least 3 ABC families (B, C, and G). Claims were staked and informal agreements to work cooperatively were made by some of the established groups. Some chose to work on specific ABC families, others on ABC transporter associated diseases, or on ABC transporters found in different organisms or organelles. An international ABC transporter research community grew and continues to thrive.

The initial fascination with P-gp was the question of how a single protein could account for resistance to so many structurally diverse anticancer drugs, an MDR phenotype. Did it involve a direct mechanism of a transporter interacting with a diversity of substrates, which was difficult to imagine at the time, or was some indirect mechanism involved, perhaps a common modifying ligand or a general modulation of membrane permeability. This key question was not settled until almost 20 years later by Adam Shapiro who used purified plasma membrane vesicles to show that P-gp could indeed function as an ATP-dependent multidrug pump (*Eur. J. Biochemistry 1997*, 250:115–121; 122–129; 130–137).

What became evident then was that a classical Michaelis–Menten kinetics understanding of substrate enzyme interaction may not be the best way to describe how a multi-spanning membrane protein such as P-gp, a molecule that spans both hydrophobic and hydrophilic domains in a complex biological membrane, interacts with its substrate. For example, the concept of substrate specificity may need to be considered in the context of the milieu in which the interaction takes place. We speculated that amphipathic compounds partitioned in the lipid bilayer may be present in high concentrations and the need for a high affinity or low Km binding may not be required; whereas molecules interacting with the P-gp molecule in a hydrophilic milieu may interact in a more classical way. This potential interplay between different domains may greatly increase the scope of the types of molecules ABC transporters are able to recognize and transport.

Recent elegant structural studies using purified proteins have resulted in much progress being made in understanding the mechanism of action of P-gp and related molecules. In particular they have provided insights into the coupling of ATP binding and hydrolysis with the transport function. However, our understanding of substrate specificity dictated in large part by the transmembrane domain is still at an embryonic stage. It is expected that new technologies such as using detergent-free extraction and reconstitution of purified ABC proteins or high resolution imaging of single molecules using cryo-EM will provide further insights into structure–function relationships of this fascinating class of transporter molecules. It is unlikely that a single structure–function mechanistic model for ABC transporters will emerge. Taking the MDR phenotype as an example, it has arose independently in three different families in the human ABC transporter superfamily. So structural similarity per se is not predictive of a phenotype. Therefore, a multidisciplinary

approach (structural, biochemical, genetics, and physiological) will be required to research each ABC transporter in detail in order to understand its biological role.

Understanding the normal or abnormal physiological function of any ABC transporter in the context of the whole organism presents another level of challenge. Gene knockout experiments in mice not infrequently result in animals with no obvious defect. This has always been puzzling given the fundamental role ABC transporters are thought to play. However, once the right stress is applied to the animal, the essential physiological role of the ABC transporter may be revealed. In other instances, compensatory mechanisms may mask the deficiency of the original knockout animal. This was the case with the bile salt export pump (Bsep, ABCB11) knockout mice, where a compensatory increased expression of mdr1a (P-gp) allowed for bile flow and the mice presented with a relatively mild non-progressive cholestasis. When the P-gp genes were knocked out in the Bsep -/- mice, a lethal, severe phenotype then ensued. These types of experiments suggest that the physiological role of a particular ABC transporter may need to be understood not as an isolated single entity but at a systems level of collaborating transporters with complementary or redundant functions. How such putative systems are regulated are not understood. In a similar vein, current worldwide efforts in whole genome sequencing will likely reveal association of ABC transporters with human diseases which may not have been anticipated. It will only be by a detailed investigation of the biochemistry, physiology, and genetics of the pathology will we get an understanding of the true function of the ABC transporter in the disease context.

Recently I visited an old gold rush town of Barkerville in northern British Columbia. At the height of the gold rush in the 1860s, Barkerville had a population of close to 20,000 people and represented one of the largest cities west of Chicago. Soon the gold rush was over and the obvious rich veins had been mined. However even today, 150 years later, gold nuggets (occasionally large and very valuable ones) continue to be discovered by individuals with modern metal detecting technology carefully going over areas that were thought to have been mined out. What is the analogy with the ABC transporter field? Another “gold rush” may occur, but in the meantime I am betting that valuable nuggets will be discovered, some of them are presented in this book.

Dr. Victor Ling, OC, OBC, FRSC
President and Scientific Director
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Distinguished Scientist, British
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Introduction

This new book on ABC transporters represents a milestone in research into this important family of membrane proteins that are engaged in either the import or export of substrates across all species. Many are responsible for multidrug resistance and a number of inherited or acquired disorders in humans. The acquired diseases are the result of either dysfunctional proteins or overexpression under certain stimuli. ABC transporters hydrolyze ATP to power a vast array of allocrites across cell membranes. They are found in all living organisms and comprise one of the largest protein superfamilies. As the chapters of this book unfold, the reader will find that ABC transporters are engaged in multifarious physiological and pathological processes, including multidrug resistance exhibited by cancers, parasites, and pathogenic microbes, immune responses, stem cell differentiation, vision, lipid and cholesterol metabolism and degenerative proteopathies.

Though we now have over a dozen complete X-ray resolved structures, we are still probing for the mechanistic suite of steps that govern the function of these proteins. It is only through the complete solving of the mechanism that we will be positioned to design inhibitors or correct mutations to the most important members of the family, that is, those that impinge on human health and well-being.

This book contains chapters on all of the important members of the ABC transporter superfamily, and we are pleased to record that many of the most eminent practitioners of research into these proteins are represented including Suresh Ambudkar, Susan Bates, Richard Callaghan, Peter Chiba, Robert Ford, Michael Gottesman, László Homolya, Toshihisa Ishikawa, Ian Kerr, Michael Otto, Bert Poolman, Balázs Sarkadi, John Schuetz, Markus Seeger, Dirk-Jan Slotboom, Gergely Szakács, and Hendrik W. van Veen. Victor Ling, the author of the *Foreword* to this book, began this journey with his discovery and naming of human P-glycoprotein, in the mid-1970s. He is clearly one of the most knowledgeable and eminent researchers of ABC transporters and a mentor to many others and their careers. He describes the last 40 years of research and the major milestones, successes and pitfalls along the way, and looks to the future. The reader will find all of the past and present leading researchers in this book, either as authors or through the many references that cite the work of these researchers.

The book is divided into two parts. In Part I, *A Structure-Function Perspective*, Chapters 'ABC Importers' and 'Bacterial ABC Multidrug Exporters: From Shared Proteins Motifs and Features to Diversity in Molecular Mechanisms' are devoted to an overview of the two major types of ABC transporters, importers, and exporters. ABC importers are found mostly in prokaryotes, and these importers employ an extracellular substrate-binding protein that captures substrate in a manner not unlike the "Venus Fly Trap" effect and presents it the membrane domains of the importer, sometimes via fused extracytoplasmic extensions that serve as substrate capture subdomains. A third type of importer, energy-coupling factor transporters, employs a membrane-embedded component to capture substrate. All three make use of the "alternating access" mechanism, as do ABC exporters, through which substrate is flipped from the outside to the inside, or vice versa, by conformational "clothes peg-like" redeployments of the two halves of the transmembrane domain that result in alternate inward- and outward-facing conformations. The whole process is choreographed by the alternating binding and hydrolysis of ATP in each of the two cytosolic ATP-binding cassette nucleotide-binding domains. ABC transporters take their family acronym name from these signature subdomains (*A*TP-*b*inding *c*assette). Chapters 'ABC Importers' and 'Bacterial ABC Multidrug Exporters: From Shared Proteins Motifs and Features to Diversity in Molecular Mechanisms', though mostly concerning bacterial importers and exporters, bring the reader well into the world of ABC transporters, including substrate diversity, multidrug efflux capacity, alternating access functionality, and evolutionarily conserved architectural membrane and cytosolic elements. Chapter 'Export of Staphylococcal Toxins by a Conserved ABC Transporter' gives us an example of pathogenesis and infection in the way that a Staphylococcal ABC exporter secretes toxins.

Chapter 'ABC Exporters from a Structural Perspective' looks at ABC exporters from a structural perspective, and in this chapter, we are taken into the present conundrum of the ABC mechanism. How exactly do they work? There are two schools of though about the type and positions of substrate-binding sites in the membrane subdomains of importers versus exporters. Additionally, there is controversy regarding the way in which the "two-cylinder engine" allosteric ATP-binding cassette dimer works. Is it like the clashing rocks of Greek mythology that threatened Jason's ship, the Argonaut, with the ATPs alternately hydrolyzing in the closed dimer, then separating to allow egress of products and reloading of ATP? Or, do these engines work in a constant contact model, with the cassettes in contact at all time, with one site primed for hydrolysis, while the other swings open to release ADP and Pi? This chapter gives us photographic snapshots of ABC exporters in various stages of the mechanism, but the complete "film" of the process lacks several important images. The final chapter of this section gives us a look at new tools of investigation that show great promise in unraveling ABC transporter biology in the wider sphere of the cell cycle.

Part II, *Human ABC Transporters of Medical Relevance*, comprises ten chapters that are devoted to ABC transporters that cause serious medical syndromes in humans. Chapters 'Genetic Polymorphisms of P-glycoprotein: Echoes of Silence'–'Just How and Where Does P-glycoprotein Bind All Those Drugs?', and

'Mechanistic and Pharmacological Insights into Modulation of ABC Drug Transporters by Tyrosine Kinase Inhibitors' examine different aspects of human P-glycoprotein (aka: ABCB1; MDR1), the "mother of all transporters," known to bind, modulate, or export well over 400 diverse compounds, including cytotoxic drugs, antibiotics, dyes, detergents, malarial drugs, lipids, steroids, glycosides, peptides, immunosuppressive agents, and others. Though there are many identified inhibitors, virtually all are ineffective in the clinic, due either to toxicity or pharmacological nuances, such as distribution, metabolism, and elimination. The overexpression and mechanism of action of P-glycoprotein has perplexed, indeed haunted, researchers for four decades. Chapter 'Genetic Polymorphisms of P-glycoprotein: Echoes of Silence' summarizes and discusses 15 years of research into genetic polymorphisms of ABCB1, and the impact of these on protein folding, drug transport, disease risk factors, and drug pharmacokinetics. Meta-analyses of ABCB1 polymorphism studies have been carried out in attempts to draw more consistent conclusions. Among ABCB1 SNPs, the synonymous C3435T polymorphic site is perhaps one of the best-known silent mutations in the field of pharmacogenomics, and consequently, it is discussed in some detail. Chapters 'Interaction of ABC Transporters with Drugs', 'Just How and Where Does P-glycoprotein Bind All Those Drugs?', and 'Mechanistic and Pharmacological Insights into Modulation of ABC Drug Transporters by Tyrosine Kinase Inhibitors' continue the theme of the interaction of ABC transporters with drugs and modulators, with P-glycoprotein at the vanguard of these studies. The *hydrophobic vacuum cleaner* and *drug flippase* models have been generated to describe P-glycoprotein's enigmatic drug-binding property. The majority of data supports the presence of a large binding domain that contains individual sites for drug interaction, linked by an intricate allosteric network that is in close communication with the ATP hydrolytic machinery. Here again, we are drawn in to this intriguing protein's structure–function anomaly. A second human P-glycoprotein (MDR2) is remarkably conserved with its partner MDR1, yet the former is not a multidrug transporter. Though a medium resolution structure of ABCB1 has been available since 2009, we are no closer to resolving the vicissitudes of this remarkable protein.

Chapters 'The ABCG2 Multidrug Transporter' and 'Human ABC Transporter ABCC11: Looking Back Pioneers' Odyssey and Creating a New Path Toward Clinical Application' concentrate on another major human efflux drug transporter, ABCG2, which is expressed at high levels in the vascular endothelium of the brain and pumps xenobiotics and chemotherapeutics back into the bloodstream. ABCG2 expression is found at high levels in some cancer types including pancreatic and liver cancers. ABCG2 has also been implicated in gout. ABCB6 is introduced in Chapter 'Human ABC Transporter ABCC11: Looking Back Pioneers' Odyssey and Creating a New Path Toward Clinical Application'. This transporter causes rare and undefined changes that affect eye development or pigmentation. Many genetic variants are discussed in these chapters, with those that lead to pathological conditions given emphasis, including potential correction strategies for prevention of treatment. Chapter 'Two Liver Transporters, ABCB11 and ABCC6; Novel Therapeutic Approaches in the Related Disorders' looks at two liver transporters,

ABCB11 and ABCC6, and at novel therapeutic approaches to correcting disorders caused by the bile salt exporters ABCB11 and another hepatic transporter ABCC6, whose function is not fully understood.

Chapter 'Biology of Mitochondrial ABCs and Their Contribution to Pathology' looks at the biology of mitochondrial ABC transporters and their contribution to pathology, including anemia, ataxia, and ischemia. Through modulation of iron and heme biosynthesis, all four mitochondrial ABC transporters (ABCB6, ABCB7, ABCB8, and ABCB10) have important roles in protecting cells against excessive reactive oxygen species. The rapid growth of personalized medicine is being supported by emerging new technologies together with accumulating knowledge of pharmacogenomics, also discussed in earlier chapters. In Chapter 'Human ABC Transporter ABCC11: Looking Back Pioneers' Odyssey and Creating a New Path Toward Clinical Application', a new SNP-typing method for the human *ABCC11* gene is expected to provide a practical tool for clinical diagnosis of axillary osmidrosis. This is the first comprehensive report on this rarely studied ABC transporter.

Chapter 'ABCC7/CFTR' introduces the cystic fibrosis transmembrane conductance regulator (CFTR), also known as ABCC7. It is an unusual member of the ATP-binding cassette family in that it is an ion channel rather than a transporter. Cystic fibrosis is caused by a multitude of inherited genetic lesions, but one in particular, the F508 deletion, responsible for over 75 % of cases. Cystic fibrosis is the commonest genetic disorder in Caucasians, though why it is so restricted remains a mystery. CFTR dysfunction affects chloride efflux in all tissues, but most seriously in the lungs, where shrinkage of the airway surface liquid layer leads to a rise in the viscosity of the mucus. The sticky mucus becomes the site of persistent bacterial and fungal infections and inflammation, leading to serious life-threatening disability. Chapter 'ABCC7/CFTR' takes a structural approach in trying to decipher the vagaries of this protein's ion channel function and dysfunction. A therapeutic drug is now available for one type of CFTR lesion, affecting about 4 % of patients.

This *Introduction* has given a brief overview of the intricacies of the ABC transporter superfamily and its especial relevance to human disease. The field is now 40 years old, but research is making such progress that we hope to enjoy the prospects of successes in combating the many identified conditions well before the next 40 years.

Anthony M. George
Peter M. Jones

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Part I
ABC Transporters: A Structure-Function Perspective

ABC Importers

Lotteke J.Y.M. Swier, Dirk-Jan Slotboom and Bert Poolman

Abstract Most ABC importers employ a soluble substrate-binding protein (SBP) to capture the ligand and donate the molecule to the translocator. The SBP can be a soluble periplasmic protein or tethered to the membrane via a lipid moiety or protein anchor or fused to the translocator. In the hybrid ABC transporters, multiple substrate-binding domains (SBDs) can be fused in tandem and provide several extracytoplasmic substrate-binding sites. The substrate is transferred from the SBP to the membrane domain, which translocates the substrate via alternating access of a membrane-embedded substrate-binding pocket. A subset of ABC transporters, known as the energy-coupling factor (ECF) transporters, employs a membrane-embedded S-component to capture the substrate. The S-component guided by the ECF module transports the substrate over the membrane via a so-called toppling mechanism. An overview of the mechanisms of transport by the different types of ABC importers is presented, together with structural information about the proteins.

Introduction

ATP-binding cassette (ABC) proteins serve many functions, including the transport of nutrients into the cell, transport of compounds across organellar membranes, the secretion of proteins, antigen (peptide) presentation, cell volume regulation, regulation of protein synthesis, detoxification, and antibiotic resistance. The vast majority of ABC proteins are part of complexes that mediate transport of molecules across cellular or organellar membranes. A smaller group of ABC proteins is associated with soluble (supra) molecular complexes and involved in DNA repair, recombination, chromosome condensation and segregation, and translation elongation. Regardless

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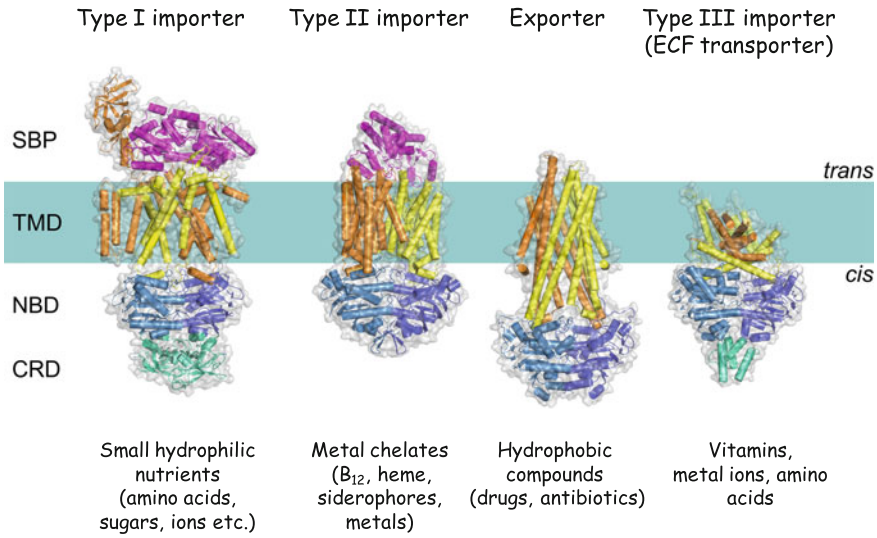

Fig. 1 ABC superfamily of transporters: Type I, II, and III importers and exporters. The TMDs are colored *orange* and *yellow*, the NBDs *light* and *dark blue*, and additional domains which often have a regulatory function, are colored *green*. The SBPs are in *purple*. From Ter Beek et al. (2014)

of whether the ABC proteins are found in membrane transport or soluble (supra) molecular complexes, they provide a power stroke in which chemical energy is converted into work (e.g., for a translocation or dislocation event).

ABC transporters are subdivided into importers and exporters. Both importers and exporters consist of two transmembrane domains (TMDs) and two cytoplasmic nucleotide-binding domains (NBDs), which power the transport through hydrolysis of ATP. The TMDs and NBDs together form the translocator. In this chapter we focus on ABC importers, which on structural and mechanistic grounds are subdivided into Type I, II, and III importers (Fig. 1) (Ter Beek et al. 2014). The mechanism of transport of Type I and II ABC importers involves the binding and release of substrate from a dedicated extracytoplasmic substrate-binding protein (SBP) and alternating access of the substrate-binding pocket in the translocator domain. Type III importers, also named energy-coupling factor (ECF) transporters, capture ligands via so-called S-components, which are small integral membrane proteins that associate with a transmembrane coupling protein and two NBDs to form a full transporter; the different types of modularity are presented in Box I. The mechanism of transport of Type III importers may involve substrate translocation by toppling of the S-component rather than alternating access of the translocator domain. The current status of the mechanisms of transport by Type I, II, and III importers is presented in separate sections below, but we first introduce the substrate-binding proteins associated with Type I and II importers and a recently proposed classification of these important accessory proteins.

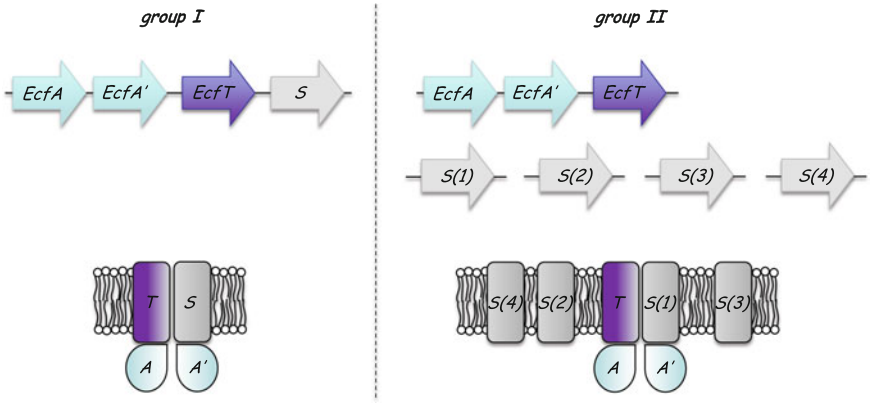

Fig. 2 Schematic representation of group I and group II ECF transporters, showing both the chromosomal location of the genes as well as the architecture of the transporters. After Slotboom (2014)

Box I: Classification within Type III ABC importers
Like the Type I and Type II ABC importers, the Type III importers consist of two identical or highly similar cytoplasmic NBDs (EcfA and EcfA') and two TMDs. One of the TMDs is called the S-component and functions as the substrate-binding protein. The other three subunits form the eponymous ECF module and fuel transport by ATP binding and hydrolysis. Based on the chromosomal location of the genes encoding all four subunits, the ECF transporters are subdivided into group I and group II. In group I transporters, the genes encoding all four subunits are present in one operon and under the control of a single promotor; the gene products form a dedicated transporter. In group II transporters, the genes for the ECF module are clustered together, while genes encoding S-components for different substrates are scattered around the genome. These S-components are all able to interact with the same ECF module. This modularity is analogous to the promiscuity of a subset of Type I importers that can interact with different SBPs or have multiple different SBDs fused to their TMD (Fig. 2).

Structural Features of Substrate-Binding Proteins

Substrate-binding proteins were first discovered in the periplasm of *Escherichia coli* (Berger and Heppel 1974), a Gram-negative bacterium, and are still often referred to as periplasmic binding proteins. A recent proteomics study showed that up to 80 % of the proteins of the periplasm of *E. coli* can be SBPs depending on the

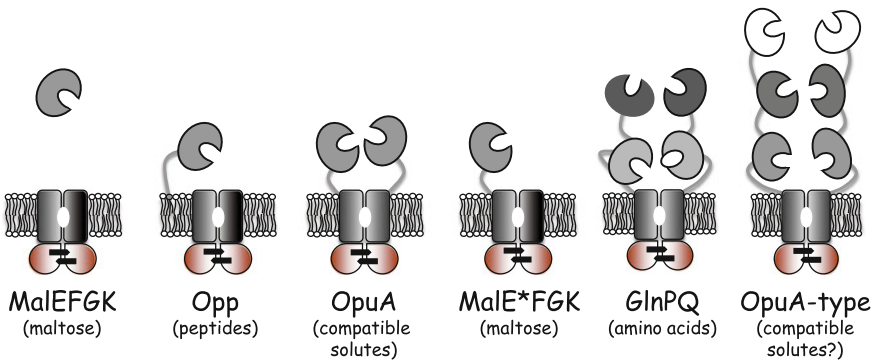

Fig. 3 Types of Type I ABC importers. The TMDs are colored *gray* and the associated SBPs or covalently linked SBDs are shown in different *shades of gray* (from *white* to *dark gray*); the NBDs are colored *orange* and two molecules of ATP are indicated by bars. After van der Heide and Poolman (2002)

growth conditions, with the number of most SBPs increasing with decreasing growth rate (Heinemann et al., unpublished). Thus, cells express more SBPs when nutrient conditions become harsh. In a few cases a limited number of related SBPs can associate with the same translocator (Davidson et al. 2008), enabling the system to import multiple distinct substrates. The first SBP crystal structure, the L-arabinose-binding protein (ABP), was solved in 1974 (Quiocho et al. 1974).

The soluble SBPs of Type I importers are present in large excess over the translocator complexes in the membrane, allowing efficient capture of substrates and initiation of the translocation reaction (Heinemann et al., unpublished). In contrast, the SBPs of Type II importers appear to be stoichiometric with the translocators. In Gram-positive bacteria and archaea, i.e., microorganisms lacking an outer membrane and periplasm, SBPs are exposed on the cell surface and attached to the cytoplasmic membrane via a lipid anchor or a transmembrane peptide (observed in archaea only), or they can be fused to the TMDs. The latter has been observed in hetero- and homodimeric TMDs, and thus results in one or two SBDs per functional complex (van der Heide and Poolman 2002; Schuurman-Wolters and Poolman, unpublished). In some cases, two or even three SBDs fused in tandem are linked to the TMDs and these systems have a total of four or six extracytoplasmic substrate-binding sites (Fig. 3). Systems with SBDs fused to the TMDs can also be found in Gram-negative bacteria but less frequently compared to Gram-positive bacteria (van der Heide and Poolman 2002). The linking of SBDs to the membrane or the fusion of multiple SBDs to the TMD increases the effective concentration of the substrate-binding sites near the translocator and increases the efficiency of transport (Schuurman-Wolters and Poolman, unpublished). The relatively short (10–20 amino acid) flexible linkers allow the SBDs to probe a small volume around the translocator, leading to an effective concentration of the SBDs in the millimolar range. The affinity of the fused SBDs for the translocator is not known, but the dissociation constants might be millimolar or higher. In case of

soluble or lipid-anchored SBPs, the affinity for the translocator is ~0.1 mM when the proteins are in the closed-liganded state (Prossnitz et al. 1989; Dean et al. 1992; Doeven et al. 2004). These low affinities may necessitate a high concentration of SBPs in the periplasm [the maltose-binding protein reaches a concentration of ~1 mM; Manson et al. (1985)] or surface-tethering of the proteins. Given that the periplasm is highly crowded and diffusion is slow (diffusion coefficients for proteins of the size of SBPs in the periplasm are even lower than in the cytoplasm; van den Berg et al., unpublished) and synthesizing a large excess of SBPs is costly, it is perhaps surprising that the covalent linking of SBDs is not more widespread or even universal for ABC importers. The mechanism of substrate binding of periplasmic, membrane-anchored, and TMD-fused SBDs is similar and so is the mode of action of the corresponding ABC transporters (vide infra).

SBPs consist of two lobes connected by a linker or hinge (domain). The two lobes close and engulf the ligand upon substrate binding (mode of substrate capture akin that of a Venus's Flytrap) (Quiocho and Ledvina 1996). Structures are available of SBPs in the open-unliganded (O), closed-unliganded (C), open-liganded (OL), and closed-liganded (CL) forms; the latter conformation is thought to productively interact with the translocator complex. Mutational and structural analyses indicate that each lobe of the SBP binds to one of the TMDs (Hollenstein et al. 2007a; Davidson et al. 2008), and conformational changes in the NBDs upon binding of ATP are transmitted via the TMDs to the SBP. Thus, ATP is involved in the opening of the SBPs and substrate transfer to the translocator.

SBPs associated with ABC transporters are related to similar domains present in a wide variety of translocation and signal transduction systems in both prokaryotic and eukaryotic organisms, including tripartite ATP-independent periplasmic (TRAP) transporters, two-component regulatory systems, guanylate cyclase–atrial natriuretic peptide receptors, G-protein coupled receptors (GPCRs), and ligand-gated ion channels (Berntsson et al. 2010). In addition, SBDs are part of prokaryotic DNA-binding proteins involved in gene regulation. SBPs are very diverse in sequence, and phylogenetic analyses based on multiple sequence alignments do not yield stable alignments (the sequence identity of the proteins is often < 20 %). However, the structures of SBPs are remarkably similar, which has been used to cluster the SBPs based on structural similarity instead of sequence similarity (Berntsson et al. 2010).

Pairwise structural alignments of more than 100 SBPs have been used to produce a structural distance tree. The SBPs were found to group into six defined clusters (A–F), three of which (clusters A, D, and F) were further subdivided. The analyses have shown that the proteins within the six clusters can be discriminated on the basis of the linker (hinge) region that connects the two protein lobes (Fig. 4): the generic secondary structure is $(\beta)_{4/5}(\alpha)_n$-hinge-$(\beta)_{4/5}(\alpha)_n$ (Fukami-Kobayashi et al. 1999). There is little or no correlation between the structural clustering and functional classification, that is, SBPs with very different substrate specificities are present in each cluster. Furthermore, in the individual clusters, the proteins are not necessarily homologous, as judged from the absence of significant sequence

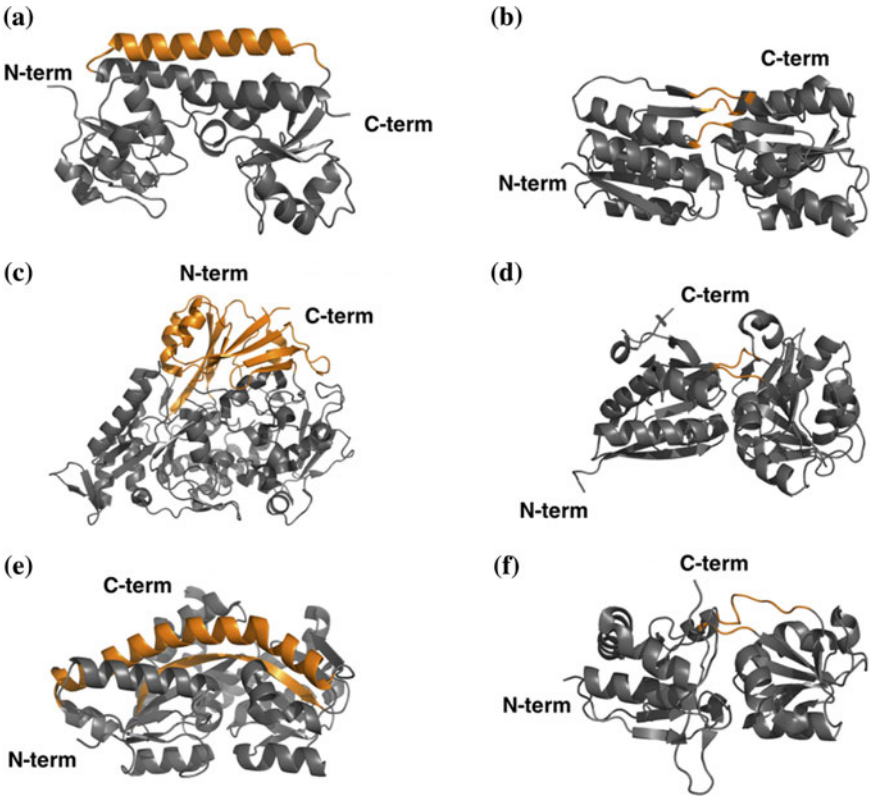

Fig. 4 The different clusters of SBPs are shown with their distinct structural features colored in *orange*. **a** Cluster A contains SBPs having a single connection between the two domains in the form of a rigid helix. **b** Cluster B contains SBPs with three interconnecting segments between the two domains. **c** Cluster C contains SBPs that have an extra domain and are significantly larger in size when compared with the others. **d** Cluster D contains SBPs with two relatively short hinges. **e** Cluster E contains SBPs associated with TRAP transporters which all contain a large helix functioning as the hinge region. **f** Cluster F contains SBPs with two hinges similarly like those of cluster D, however, these hinges have almost double the length creating more flexibility inside the SBP. Please note that Clusters A, D, and F can further be subdivided based on the substrate of the SBP (see text). The proteins used to illustrate the features in Cluster A–F are BtuF (PDB code: 1N2Z), RBP (PDB code: 1DRJ), OppA (PDB code: 3DRF), ModA (PDB code: 1ONR), UehA (PDB code: 3FXB), and HisJ (PDB code: 1HSL), respectively. Figure was taken from Berntsson et al. (2010)

similarity. Clusters B and F contain the most diverse SBPs, associated with various types of transport and signal transduction systems; SBPs in cluster A are unique to type II ABC importers; clusters C and D contain SBPs of Type I ABC importers; and SBPs in cluster E are found in TRAP transporters. Specific information on the six clusters is presented in Box II.

Box II: Structural features distinguishing substrate-binding proteins

Cluster A: The cluster A SBPs associate with Type II ABC importers. The distinguishing characteristic of these SBPs is an α-helix that serves as a hinge between the two domains. The rigidity of this helix is reflected in the small movement of both domains upon substrate binding. All of the SBPs in cluster A play a role in metal binding, either directly or as metal chelates.

Cluster B: Cluster B consists of SBPs that bind carbohydrates, branched chain amino acids, natriuretic peptides, and autoinducer-2 (AI-2). The SBPs in cluster B interact with type I ABC importers, two-component histidine-sensory complexes, and guanylate cyclase-atrial natriuretic peptide receptors. The hinge of the SBPs in cluster B is built of three distinct regions connecting the lobes. Homologous to the proteins in cluster B are the *lac*-repressor type transcription factors.

Cluster C: The cluster C SBPs interact with Type I ABC importers and bind diverse ligands including di- and oligopeptides, arginine, nickel ions, and cellobiose. They all have an extra domain, which in oligopeptide transporters extends the binding cavity to accommodate very large ligands (peptides of 20 residues or more).

Cluster D: The discernible feature of these proteins is that their hinge region consists of two short stretches, 4–5 amino acids long. This large group of SBPs binds a large variety of substrates: carbohydrates, putrescine, thiamine, tetrahedral oxyanion as well as ferric or ferrous iron.

Cluster E: These SBPs are part of the TRAP transporter (tripartite ATP-independent periplasmic transporter) family. TRAP transporters use an electrochemical ion gradient to fuel the uphill translocation of substrates. The remarkable feature of TRAP SBPs is a large single ß-strand that is part of the two five-stranded ß-sheets of both lobes. All TRAP-dependent SBPs structurally characterized have a conserved strand order, typical of class II SBPs ($\beta_2\beta_1\beta_3\beta_n\beta_4$), and an additional ß-strand connecting both domains. A second distinguishing feature is a long helix that spans both domains, although in some structures it is interrupted by a kink. The known substrates are ectoine, pyroglutamic acid, lactate, 2-keto acids, and sialic acid.

Cluster F: The distinguishing feature of the cluster F proteins is a hinge consisting of two segments connecting the two lobes. The linker stretches of cluster F proteins are significantly longer (8–10 amino acids) than the hinges of 4–5 amino acids observed in SBPs of cluster D. Possibly the longer linker provides more flexibility between the open and closed conformation. Cluster F SBPs bind a large variety of substrates ranging from trigonal planar anions (nitrate, bicarbonate) to amino acids and compatible solutes such as glycine betaine.

Mechanism of Ligand Binding

SBPs have been captured in crystals in different conformations: open (O) and closed (C), either with or without ligand (L). In solution, the equilibrium state of the SBPs is toward the open (O) conformation in the absence of ligand and toward the closed conformation in the presence of ligand (CL). The crystal structures provide crucial snapshots of different conformational states of the SBPs, but they do not reveal the dynamics of ligand binding. Different models for ligand binding are possible. In the *induced fit* mechanism, the initial interaction between protein and ligand (OL) is relatively weak but triggers the closing of the protein and formation of CL (Fig. 5). In the *conformational selection* mechanism, the protein samples closed states (partially closed (PC) or fully closed (C) in Fig. 5) without the involvement of a ligand. Ligand binding stabilizes the PC or C-form and thereby drives the equilibrium to the closed form CL. A combination of both mechanisms is also possible, but nowadays it is thought that conformational selection is the dominant mechanism of ligand binding by enzymes and receptors (Vogt and Di Cera 2013; Clore 2014).

The two mechanisms make specific predictions about the kinetics of ligand binding, i.e., the closing rate increases with ligand concentration in *induced fit* but not in *conformational selection* (Fig. 5). Moreover, in the *conformational selection* mechanism, the life-time of the closed state increases with ligand concentration. To discriminate between *induced fit* and *conformational selection* is generally not possible in ensemble measurements because differences in conformations are averaged out. Single-molecule observations with high time resolution allow the two mechanisms or hybrid forms to be discriminated. Recent single-molecule Förster resonance energy transfer (smFRET) studies of the maltose-binding protein (MalE)

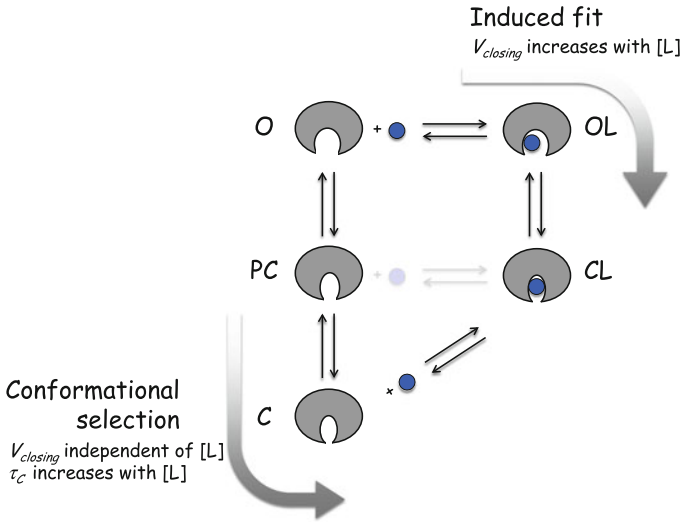

Fig. 5 Ligand-binding mechanisms. Conformational transitions in SBDs according to the induced fit or the conformational selection binding mechanism, as explained in the main text. V_{closing}, rate of closing of the binding site; τ_c, life-time of the closed state; and [L], ligand concentration

(Kim et al. 2013; Seo et al. 2014) and the two SBDs of the amino acid ABC importer GlnPQ (Gouridis et al. 2015) have shown that these proteins bind their ligands by a previously undocumented type of *induced fit* mechanism: the SBDs intrinsically transit from O to C in addition to being triggered to close by the ligand. Remarkably, as shown for both SBDs from GlnPQ, the life-time of C is the same as that of CL when high-affinity ligands (with sub-micromolar dissociation constants) are bound. In contrast, the life-time of CL is dramatically shortened when low-affinity ligands are bound. This observation indicates that the ligands do not "stabilize" the closed conformation, which is often thought on the basis of the multiple interactions between protein and ligand in crystal structures.

Box III. The GlnPQ transporter

The GlnPQ transporter from *Lactococcus lactis* and other Gram-positive bacteria has homodimeric TMDs with two SBDs fused in tandem to each of them (Fig. 3, 6th cartoon). The N-terminal SBD (SBD1) is preceded by a signal sequence that is cleaved off following translocation of the SBDs across the membrane (Schuurman-Wolters and Poolman 2005).

Thus, in GlnPQ four SBDs compete for interaction with the translocator to deliver the bound ligand. The close proximity of multiple SBDs, each of which can adopt different conformations (O, C, OL, or CL) and close intrinsically, affects transport (Gouridis et al. 2015). GlnPQ imports asparagine solely via SBD1 (K_D = 0.2 μM), and glutamine via both SBD1 (K_D ∼ 100 μM) and SBD2 (K_D = 1 μM), but with affinities that differ by two orders of magnitude (Fulyani et al. 2013). The transport of asparagine has been determined in the absence of glutamine (SBD2 ∼ 98 % in O, ∼2 % in C), in the presence of relatively low concentrations of glutamine that do not significantly compete with Asn for binding to SBD1 (SBD2 > 95 % in CL), and in the absence or presence of glutamine and a mutation (D417F) that prevents SBD2 from binding ligand and, more importantly, prevents closing (SBD2 ∼ 100 in O) (Gouridis et al. 2015). The remarkable observation was made that the D417F mutation increases the rate of asparagine import by a factor of 3, showing that 2 % of C already has a strong inhibitory effect on transport via SBD1. In fact, the inhibition of asparagine transport via SBD1 by 2 % of SBD2 in the C state is comparable to the inhibition by 95 % in the CL.

These experiments indicate that the TMDs interact with the closed state of the SBD, but the interaction is shorter-lasting for CL (leading to a productive translocation cycle) than for C (leading to a long-lived blocked intermediate state). This work illustrates the importance of understanding the mechanism of ligand binding to substrate-binding proteins in order to begin to understand the full translocation cycle. Although not so easily probed in ABC importers with periplasmic or lipid-anchored SBPs, similar kinetics of ligand binding and competition between proteins in different conformation states could play a role in these systems.

Structural Features of ABC Importers

Type I Importers

Crystal structures of Type I importers for four different substrates are available: the molybdate/tungstate transporter $ModB_2C_2$ from *Archaeoglobus fulgides* (Hollenstein et al. 2007b) and *Methanosarcina acetivorans* (Gerber et al. 2008), the maltose transporter $MalFGK_2$ from *E. coli* (Oldham et al. 2007, 2013; Khare et al. 2009; Oldham and Chen 2011a, b; Chen et al. 2013), the methionine transporter $MetN_2I_2$ from *E. coli* (Kadaba et al. 2008; Johnson et al. 2012), and the transporter for basic amino acids $Art(QN)_2$ from *Thermoanaerobacter tengcongensis* (Yu et al. 2015). $MalFGK_2$ is by far the most studied transporter. The different crystal structures are listed in Table 1.

The crystal structure of $MalFGK_2$ in the inward-facing apo-state is likely representing the resting state of the transport cycle (Khare et al. 2009). The two TMDs of $MalFGK_2$, MalF and MalG, are not identical. They consist of eight and six transmembrane helices, respectively, from which TM3-8 of MalF are related to TM1-6 of MalG. These helices form the core region of the TMDs with pseudo-twofold symmetry. In the inward-facing state, the interface between MalF and MalG creates a translocation cavity, which is accessible from the cytoplasm. On the periplasmic end, this cavity is closed by a periplasmic, hydrophobic gate that is composed of four loops, each at the kink of a transmembrane helix (TM5 and TM7 from MalF, TM3 and TM5 from MalG). Both MalF and MalG have a coupling helix, which is located in loop L6 between TM6 and TM7 of MalF and in loop L4 between TM4 and TM5 of MalG. Both coupling helices dock into a groove on the surface of each of the two NBDs (MalK subunits). These grooves are lined by two helices from the helical subdomain of MalK, the helix following the Walker A motif and residues from the Q-loop. The interactions between the coupling helices and the MalK subunits transduce conformational changes upon ATP binding and hydrolysis to MalF and MalG and allow alternate access of the binding pocket in the TMDs.

Within the MalF–MalG dimer, an occluded maltose-binding pocket is formed about halfway the membrane. In the structures showing maltose-bound $MalFGK_2$ in a pre-translocation state (Oldham and Chen 2011a), this pocket is closed at the periplasmic side by a hydrophobic gate. On the cytoplasmic side, the pocket is closed by a network of van der Waals interactions. The binding pocket is lined by residues from MalF only, and the binding mode resembles that of MBP in terms of aromatic stacking of binding site residues with the sugar rings, as well as a hydrogen-bond network involved in sugar recognition.

A crystal structure of nucleotide-free $MalFGK_2$–MBP in the pre-translocation state with maltoheptaose bound has shed light on the selectivity of sugar transport (Oldham et al. 2013). $MalFGK_2$ imports linear malto-oligosaccharides of a length from two to seven glycosyl units, linked through α-1,4 glycosidic bonds, with an unmodified reducing end. In the crystal structure, four glycosyl units from the

Table 1 The different crystal structures available of ABC importers

Name (organism)	Remarks	Substrate bound	Resolution (Å)	PDB code	Reference
Type I importers					
Molybdate transporter $ModB_2C_2$ in complex with ModA (*Archaeoglobus fulgidus*)	Inward-facing conformation, with SBP	Mg^{2+}, PO_4^{3-}, WoO_4^{2-}	3.10	2ONK	Hollenstein et al. (2007a, b)
Molybdate ABC transporter ModBC in a *trans*-inhibited state (*Methanosarcina acetivorans*)	Inward-facing conformation, without SBP bound	Mg^{2+}, WoO_4^{2-}	3.00	3D31	Gerber et al. (2008)
Maltose transporter $MalFGK_2$ in complex with MBP (*Escherichia coli*)	Outward-facing conformation stabilized by a mutation in the NBDs (MalK E159Q)	Maltose, ATP	2.80	2R6G	Oldham et al. (2007)
Maltose transporter $MalFGK_2$ (*E. coli*)	TM helix 1 deleted, in inward-facing conformation, in resting state, without MBP	–	4.50	3FH6	Khare et al. (2009)
Maltose transporter $MalFGK_2$ in complex with MBP (*E. coli*)	Pre-translocation intermediate state, with MBP	AMP–PNP, Mg^{2+}, maltose	2.90	3PUZ	Oldham and Chen (2011a)
After crystal soaking	Pre-translocation, outward-facing conformation, with MBP	AMP–PNP, Mg^{2+}, maltose	3.10	3PUY	
With mutations in MBP	Pre-translocation intermediate state with mutations in MBP (G69C/S337C) that stabilize the substrate-bound closed conformation by a crosslink	Maltose	3.10	3PV0	
Maltose transporter $MalFGK_2$ in complex with MBP (*E. coli*)	Outward-facing conformation with MBP	AMP–PNP, Mg^{2+}, maltose	2.20	3RLF	Oldham and Chen (2011b)
With orthovanadate	Outward-facing conformation, ADP in conjunction with vanadate, with MBP	ADP · VO_4^{3-}, Mg^{2+}, maltose	2.40	3PUV	
With tetrafluoroaluminate	Outward-facing conformation, ADP in conjunction with tetrafluoroaluminate, with MBP	ADP · AlF_4^-, Mg^{2+}, maltose	2.30	3PUW	
With beryllium trifluoride	Outward-facing conformation, ADP in conjunction with beryllium trifluoride, with MBP	ADP · BeF_3^-, Mg^{2+}, maltose	2.30	3PUX	
			3.91	4JBW	

(continued)

Table 1 (continued)

Name (organism)	Remarks	Substrate bound	Resolution (Å)	PDB code	Reference
Maltose transporter $MalFGK_2$ in complex with MBP (*E. coli*)	Complex with regulatory protein $EIIA^{glc}$, inward-facing conformation				Chen et al. (2013)
Maltose transporter $MalFGK_2$ in complex with MBP (*E. coli*)	Pre-translocation conformation; maltoheptaose bound	Maltoheptaose	2.90	4KHZ	Oldham et al. (2013)
	Pre-translocation, outward-facing conformation	ANP, α-D-glycose	2.38	4KI0	
Methionine transporter $MetN_2I_2$ (*E. coli*) (dodecylmaltoside-purified)	Inward-facing conformation	–	3.70	3DHW	Kadaba et al. (2008)
At higher resolution (Cymal5-purified)	Inward-facing conformation	ADP	2.90	3TUI	Johnson et al. (2012)
(decylmaltoside-purified)	Inward-facing conformation, C2 domains repositioned	–	4.00	3TUJ	
Se-Methionine soaked (Cymal5-purified)	Inward-facing conformation	ADP	3.40	3TUZ	
Amino acid transporter $Art(QN)_2$ (*Thermoanaerobacter tengcongensis*)	Inward-facing conformation	–	2.80	4YMS	Yu et al. (2015)
With arginine	Inward-facing conformation	Arginine	2.59	4YMT	
With ATP and arginine	Inward-facing conformation	ATP, arginine	2.50	4YMU	
With ATP	Inward-facing conformation	ATP	3.00	4YMV	
With histidine	Inward-facing conformation	Histidine	2.80	4YMW	
Type II importers					
Vitamin B_{12} transporter BtuCD (*E. coli*)	Outward-facing conformation, no SBP bound	$V_4O_{12}^{4-}$	3.20	1L7V	Locher et al. (2002)
With nucleotide	Outward-facing conformation	AMP–PNP	2.79	4R9U	Korkhov et al. (2014)
Vitamin B_{12} transporter BtuCD in complex with BtuF (*E. coli*)	Intermediate occluded state. The translocation pore is closed from both sides. BtuF is in an open state	–	2.60	2QI9	Hvorup et al. (2007)

(continued)

Table 1 (continued)

Name (organism)	Remarks	Substrate bound	Resolution (Å)	PDB code	Reference
Vitamin B_{12} transporter BtuCD in complex with BtuF with mutation in NBD (*E. coli*)	E159Q mutation abolished ATP hydrolysis activity, BtuC in asymmetric conformation	–	3.49	4DBL	Korkhov et al. (2012a)
Vitamin B_{12} transporter BtuCD in complex with BtuF (*E. coli*)	Intermediate state	AMP–PNP	3.47	4FI3	Korkhov et al. (2012b)
Putative metal-chelate-type ABC transporter HI1470/1471 (*Haemophilus influenzae*), later renamed $MolB_2C_2$ (when shown to bind WoO_4^{2-}/MoO_4^{2-})	Inward-facing conformation, without SBP	–	2.40	2NQ2	Pinkett et al. (2007)
Heme transporter HmuUV (*Yersinia pestis*)	Outward-facing	–	3.00	4G1U	Woo et al. (2012)
ECF-type importers					
RibU S-component for riboflavin (*Staphylococcus aureus*)	Substrate bound	Riboflavin	3.60	3P5N	Zhang et al. (2010)
ThiT S-component for thiamin (*Lactococcus lactis*)	Substrate bound	Thiamin	2.00	3RLB	Erkens et al. (2011)
BioY S-component for biotin (*Lactococcus lactis*)	Substrate bound	Biotin	2.10	4DVE	Berntsson et al. (2012)
NikM S-component for Ni^{2+} (*Thermoanaerobacter tengcongensis*)	Substrate bound	Ni^{2+}/Co^{2+}	2.50, 1.83, 3.21	4M5C, 4M5B, 4M58	Yu et al. (2014)
CbiO homodimer of CbiMNQO (*Thermoanaerobacter tengcongensis*)	Nucleotide free	–	2.30	4MKI	Chai et al. (2013)

(continued)

Table 1 (continued)

Name (organism)	Remarks	Substrate bound	Resolution (Å)	PDB code	Reference
EcfAA' heterodimer (*Thermotoga maritima*)	Nucleotide bound	ADP	2.70	4HLU	Karpowich and Wang (2013)
Folate ECF transporter (*Lactobacillus brevis*)	Substrate free	–	3.00	4HUQ	Xu et al. (2013)
Putative Pdx ECF transporter (*Lactobacillus brevis*)	Substrate free	–	3.53	4HZU	Wang et al. (2013)
Pantothenate ECF transporter (*Lactobacillus brevis*)	Substrate free	–	3.25	4RFS	Zhang et al. (2014)

reducing end of maltoheptaose are bound in the groove between the N-terminal and C-terminal lobe of MBP. A conserved glutamine in the periplasmic L5 loop of MalG is also inserted into this groove and forms hydrogen bonds with the first glycosyl unit at the reducing end. There is no clear electron density found for the glycosyl units at the non-reducing end of maltoheptaose. In a different AMP–PNP bound, pre-translocation state structure with an outward-facing conformation, three glucosyl units from the non-reducing end of maltoheptaose are bound in the MalF-binding site (Oldham et al. 2013). Aromatic stacking interactions and five direct protein–sugar hydrogen bonds within this binding site indicate specificity for α-1,4 linkage of the glycosyl units. The other glucosyl units of maltoheptaose are not visible, probably because they do not have specific interactions with the transporter. Overall, it seems that the size-exclusion limit of transport by $MalFGK_2$ is determined by the size of the cavity in the occluded state, which is about 2400 Å, just large enough to fit a maltoheptaose molecule.

Five crystal structures of the transporter for basic amino acids $Art(NQ)_2$ from *T. tengcongensis* in the apo-, arginine-, histidine-, ATP-, and ATP plus arginine-bound state all show an inward-facing conformation, with the nucleotide-binding domains (ArtNs) in a semi-open state (Yu et al. 2015). The transmembrane domains (ArtQs) contain five transmembrane segments, which correspond to TM4-8 of MalF and TM2-6 of MalG, and form a homodimer with twofold symmetry. Together, the ArtQs form a highly negatively charged tunnel reaching from the periplasmic side to the cytoplasmic side of the membrane, which allows positively charged amino acids to pass through. Halfway across the predicted membrane, each ArtQ subunit has a highly negatively charged substrate-binding pocket, lined by highly conserved residues that are involved in specific binding of both arginine and histidine. Remarkably, two amino acids are present in the ArtQ dimer, which raises questions on the transport mechanism as one amino acid at a time is transferred from the substrate-binding protein ArtI to $Art(NQ)_2$. A crystal structure of ArtI in the arginine bound state shows only one binding site. We therefore consider it more likely that the two amino acids have accessed the binding pocket when the protein was trapped in the inward-facing conformation, and that the structures do not reflect a true translocation intermediate.

Capturing $MalFGK_2$ in an ATP-bound state was achieved by mutating the catalytic glutamate in the ATP hydrolysis site to a glutamine (E159Q) (Oldham et al. 2007). In the outward-facing conformation, the translocation cavity is now open to the bound MBP, which is in a substrate-free state (Oldham et al. 2007; Oldham and Chen 2011a, b). On the cytoplasmic side, the translocation cavity is closed by a tightly packed helix bundle of TM6 and TM7 from MalF and TM4 and TM5 from MalG. In the pre-translocation, occluded state, only four residues from these TMs are forming the gate. In the closed, nucleotide-bound MalK dimer, two ATP or AMP-PNP molecules are sandwiched at the dimeric interface, where they interact with residues from the Walker A and Walker B motifs of one MalK subunit and residues from the LSGGQ signature sequence of the other subunit. Compared to the pre-translocation, occluded state, the helical domains of the NBDs have rotated another 15°, thereby breaking the intersubunit hydrogen-bond network of

the MalK dimer. Overall, when comparing the inward-facing and outward-facing conformations, the structures of the core helices TM4-7 in MalF and TM2-5 in MalG are maintained as rigid bodies during the transport cycle. The other helices, TM2, TM3, and TM8 of MalF and TM1 and TM6 of MalG, move together with the core helices of the other TMD subunit. The helical domains of the NBDs, as well as the coupling helices, rotate over an angle of about 30° upon closure of the MalK dimer, which allows the conformational change in the MalFG dimer.

$MalFGK_2$ has been crystallized in complex with the MBP, maltose, and ADP together with phosphate analogs VO_4^-, AlF_4^-, or BeF_3^-, which has provided insight into the mechanism of ATP hydrolysis within the MalK dimer (Oldham and Chen 2011b). The overall structures show the same outward-facing conformation as described above, suggesting that ATP hydrolysis does not force the transporter in a different conformation. Even the conserved residues within the ATP hydrolysis site are superimposable on all four structures. The hydrolysis of ATP proceeds by the attack of the γ-phosphate of ATP by a water molecule, which is thought to transit a pentacovalent intermediate state. The VO_4^- and AlF_4^- bound structures show a trigonal bipyramidal and octahedral geometry, respectively, which represent the transition state of the hydrolysis. Three of the four oxygen atoms of VO_4^- or the fluorides of AlF_4^- lie in the equatorial plane, while the fourth oxygen of VO_4^- or the attacking water molecule in case of the AlF_4^- bound structure and the oxygen connecting the β-phosphate group are found at the axial positions. This transition state supports the catalysis-by-a-general-base model, where the attacking water molecule is activated by the conserved glutamate (E159) by polarizing the water molecule. The conserved histidine (H192) in the H-loop positions the γ-phosphate group, the attacking water molecule, and the conserved glutamate, while the conserved glutamine (Q82) in the Q-loop is involved in coordination of the Mg^{2+} ion.

In the final step before returning to the resting state, the SBP is released from the transporter and the inorganic phosphate and ADP are released from the NBDs. Intermediate states of this step are observed in the crystal structure of the molybdate/tungstate transporter $ModB_2C_2$ from *A. fulgides,* which is in the inward-facing conformation in complex with its substrate-binding protein ModA (Hollenstein et al. 2007a, b), and two crystal structures of ADP-bound $MetN_2I_2$ in the inward-facing conformation without the substrate-binding protein MetQ bound (Johnson et al. 2012). The structure of the $ModB_2C_2$-A complex is similar to that of $MalFGK_2$ in the resting state, with the NBDs (ModC) in an open, nucleotide-free conformation and the TMDs (ModB) in an inward-facing conformation. ModC adopts a post-hydrolysis conformation and two molecules of inorganic phosphate are bound at the position of the β-phosphate groups of ATP in the ATP-bound structure of MalFGK-MBP (Oldham et al. 2007), suggesting that binding of a phosphate group at this position is probably the strongest compared to the position of the α- or γ-phosphate group.

Two pairs of crystal structures of the methionine transporter $MetN_2I_2$ from *E. coli* are available (Kadaba et al. 2008; Johnson et al. 2012), with the NBDs (MetN) spaced differently between the nucleotide-free and the ADP-bound structures. Similar to the structure of $MalFGK_2$ in the resting state, the MetN dimer is

held together by dimerization of the C-terminal domains. Each TMD contains five transmembrane segments, which correspond to TM4-8 of MalF and TM2-6 of MalG. The region of highest sequence and structure similarity is found at the region of the coupling helices and TM3 and TM4 located on either side of them. Similar to the maltose transporter, a network of salt bridges between the coupling helices of MetI and the helical subdomain of MetN relay the conformational changes between the TMD and NBD. The gate that closes the translocation cavity at the periplasmic side of $MetN_2I_2$ is structurally conserved and found in the $ModB_2C_2$ transporter.

Type II Importers

The Type II importers facilitate the uptake of metal chelates including vitamin B_{12}, heme, and oxanions (Klein and Lewinson 2011). For this group of ABC importers, seven crystal structures of complete transporters are available (Table 1). These structures correspond to five different states, which represent the major steps of the transport cycle. Five crystal structures are of the vitamin B_{12} transporter BtuCD from *E. coli* (Locher et al. 2002; Hvorup et al. 2007; Korkhov et al. 2012a, b, 2014), which is the most studied Type II importer. The two other crystal structures are of the molybdate/tungstate transporter $MolB_2C_2A$ from *Haemophilus influenzae* (Pinkett et al. 2007) and the heme transporter HmuUV from *Yersinia pestis* (Woo et al. 2012).

In 2002, the vitamin B_{12} transporter BtuCD from *E. coli* was crystallized in an outward-facing conformation, forming a cavity accessible from the periplasmic side (Locher et al. 2002). No substrate or nucleotides are bound and this outward-facing conformation probably represents the resting state of the transport cycle. The BtuC subunit consists of ten transmembrane helices, of which TM2-5 and TM7-10 are related by a pseudo-twofold rotation axis. Within the BtuC dimer, there is a twofold symmetry axis running down the translocation pathway. This translocation pathway is lined by residues from TM5, TM5a (small transmembrane helix following TM5), and TM10 of each BtuC, and the loops preceding TM3 and TM8. In this outward-facing conformation, the translocation cavity stretches out two-thirds into the predicted membrane and is closed by cytoplasmic gate I.

Given the millimolar ATP concentration in the cell, the resting state of the transporter will be short lived. The BtuDs will quickly bind ATP and the transporter will convert into an outward-facing, nucleotide-bound state as represented by the recently solved crystal structure of BtuCD in complex with two molecules of the non-hydrolyzable ATP analog AMP-PNP and two Mg^{2+} ions (Korkhov et al. 2014). Like in the resting state, the BtuC dimer is in an outward-facing conformation with a translocation cavity open to the periplasmic side but closed at the cytoplasmic side by a second cytoplasmic gate, known as cytoplasmic gate II. The BtuDs form a closed dimer with the AMP-PNP molecules and Mg^{2+} ions bound at the ATP hydrolysis site; in this state the two coupling helices of the BtuCs are about 9.5 Å closer to each other compared to the resting state. Apparently, the binding of

AMP-PNP promotes closure of the BtuD dimer, which is coupled to opening of cytoplasmic gate I and closure of cytoplasmic gate II in BtuC.

In the next step of the transport cycle, the substrate-binding protein BtuF binds vitamin B_{12} and subsequently docks onto the periplasmic side of the BtuC dimer. A study with BtuCD reconstituted in proteoliposomes has shown that ATP binding promotes docking of BtuF onto the BtuCD complex, rather than simulating BtuF release to scavenge vitamin B_{12} (Korkhov et al. 2014). Upon docking of BtuF onto the BtuCD complex, the N-terminal and C-terminal lobe of BtuF are spread apart while making interactions with periplasmic loops of BtuC. TM5a of BtuC sticks into the vitamin B_{12} binding site of BtuF, and this distortion forces vitamin B_{12} to move into the translocation cavity. Rearrangements within the BtuC dimer will then result in a substrate-bound, occluded state, which has been obtained in the presence of AMP-PNP (Korkhov et al. 2012b). In this state, the BtuD dimer closely resembles the previous state, but TM5 and TM5a of both BtuC subunits have now closed the translocation cavity from the periplasmic side, while cytoplasmic gate II is still closed. Although vitamin B_{12} is not present in this crystal structure, the cavity is wide enough to host the molecule with only minor steric hindrance. The interior of the cavity does not resemble the substrate-binding pocket of BtuF, rather it forms a low-affinity chamber.

After hydrolysis of ATP, the cytoplasmic gate II in the BtuD dimer will open to release inorganic phosphate and ADP. The substrate may then be squeezed out by a peristaltic-like movement. The state after substrate release is visualized by the crystal structure of the molybdate/tungstate transporter $MolB_2C_2$ from *Haemophilus influenzae* (Pinkett et al. 2007). The transporter was crystallized in a nucleotide-free, inward-facing conformation in the absence of the substrate-binding protein MolA. The arrangement of the transmembrane helices of MolB is similar to that of BtuC. In $MolB_2C_2$ only the TM5a of each subunit contributes to the formation of the periplasmic gate. EPR and selective crosslinking studies show that this gate offers a narrower opening upon nucleotide binding compared to the periplasmic gate in BtuCD, which is in agreement with the much smaller substrates molybdate or tungstate as compared to vitamin B_{12} (Rice et al. 2013).

To return to the outward-facing resting state, BtuF should dissociate and cytoplasmic gate I should close while the periplasmic gate needs to open up. A post-translation, intermediate state was crystallized in two different asymmetric conformations (Hvorup et al. 2007; Korkhov et al. 2012a). While the two non-identical lobes of BtuF interact with the periplasmic loops of the BtuC subunits, a slight asymmetry is introduced in the loops, resulting in an asymmetric arrangement of the transmembrane helices. Especially the orientation of TM3-5a is thought to control on which side of the membrane the translocation cavity is opened. The closure of the periplasmic gate and cytoplasmic gate I ensures that no continuous channel is formed, while switching from the inward-facing to the outward-facing conformation. Once the transporter reaches the outward-facing conformation by opening the periplasmic gate, BtuF will be released and the transporter is back in the resting state.

Type III Importers

Compared to the Type I and Type II importers, the structural information on the Type III importers/ECF transporters is limited. To date, crystal structures of four different S-components (Zhang et al. 2010; Erkens et al. 2011; Berntsson et al. 2012; Yu et al. 2014), two NBD dimers (Chai et al. 2013; Karpowich and Wang 2013), and three complete transporters are available (Xu et al. 2013; Wang et al. 2013; Zhang et al. 2014) (Table 1). The four substrate-bound S-components are RibU for riboflavin from *Staphylococcus aureus* (Zhang et al. 2010), ThiT for thiamin and BioY for biotin from *Lactococcus lactis* (Erkens et al. 2011; Berntsson et al. 2012), and NikM2 for Ni^{2+} for *Thermoanaerobacter tengcongensis* (Yu et al. 2014).

S-components. RibU, ThiT, and BioY associate with group II ECF transporters and despite a sequence identity of only 14–16 %, these S-components share a common structural fold of a six transmembrane helical bundle. The N-terminal parts consisting of TM1-3 are structurally the most conserved and were predicted to be involved in the interaction with the ECF module. TM1 contains a conserved AxxxA motif, which is important for complex formation and thiamin transport by ECF ThiT from *L. lactis* (Erkens et al. 2011) and biotin transport by the group I transporter BioMNY from *Rhodobacter capsulatus* (Finkenwirth et al. 2015). TM4-6 in the C-terminal parts of S-components are structurally more different and are the main contributors to the substrate-binding site. This combination of a structurally conserved part and a highly divergent part explains how different S-components can interact with one and the same ECF module and maintain a high specificity for chemically diverse substrates (dissociation constants in the sub-nanomolar to nanomolar range). The substrate-binding pocket is located on the predicted extracytoplasmic side of the S-component and closed by the L1 loop connecting TM1 and TM2, which lies over the pocket in a lid-like manner. EPR studies on thiamin-specific ThiT from *L. lactis* have shown that the main difference between thiamin-bound and substrate-free ThiT is the conformation of this L1 loop, covering the substrate-binding pocket in the liganded state, while exposing it to the environment in the unliganded state (Majsnerowska et al. 2013).

NikM2 is the S-component of the NikM2N2OQ transporter from *T. tengcongensis*, which is a metal transporter of the group I ECF transporters (Yu et al. 2014). Compared to the three vitamin-specific S-components, NikM2 has an additional N-terminal α-helix. The highly conserved extracellular N-terminus inserts into the center of the S-component and occupies a space that corresponds to the substrate-binding pocket in the vitamin-specific S-components. Within this space, Ni^{2+} is bound in a square-planar geometry by four nitrogen atoms. The nickel-binding residues are stabilized and oriented by a network of hydrogen bonds.

Full-length transporters. The structures of the complete Type III transporters all show the same conformation, in which the EcfAA' heterodimer adopts an open conformation and the substrate-free S-components lie in the membrane with the helical axis almost perpendicular to the plane of the membrane (Fig. 6). These

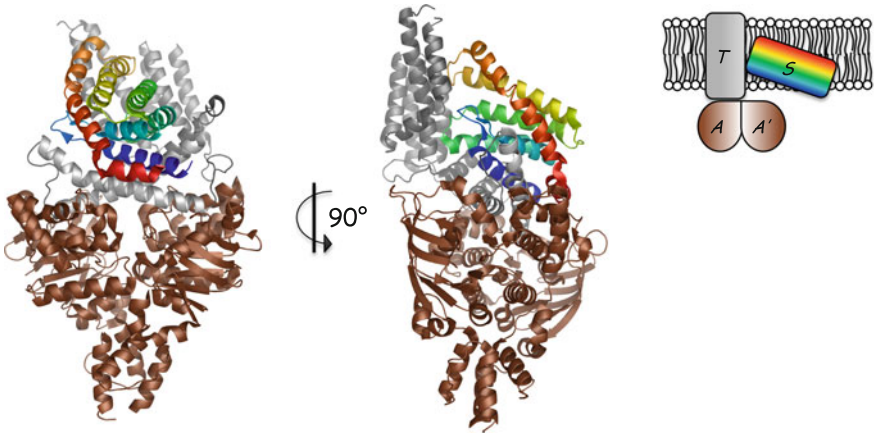

Fig. 6 Crystal structure of a Type III ABC importer. The crystal structure of ECF FolT is shown in the secondary-structure representation from the plane of the membrane from two sides, related by rotation of 90°. The two NBDs are colored in *brown*, EcfT is colored in *gray* and the S-component is colored from the N-terminus in *blue* to the C-terminus in *red*. In the *upper-right corner*, a schematic representation showing all subunits in the same color code is given to indicate the position of the membrane. The figure was generated with PyMoL, using structure PBD ID: 4HUQ

transporters have the same ECF module in complex with three different S-components: FolT for folate (Xu et al. 2013), PanT for pantothenate (Zhang et al. 2014), and HmpT; the latter is thought to bind pyridoxine (and should therefore be named PdxU) (Wang et al. 2013). The NBDs have the same structural fold as in Type I and Type II importers, including the RecA-like domain, the helical domain, and a C-terminal domain. All the conserved features (Walker A, Walker B, LSGGQ signature motif, D-, H-, and Q-loop) are present. A C-terminal extra domain provides the basis for dimerization (Chai et al. 2013; Karpowich and Wang 2013). Like the NBDs of the Type I and Type II importers, both dimers have two grooves at the interface of the RecA-like domain and the helical domain within one ATPase subunit, which can form the anchor points of eventual coupling helices. These acidic and highly conserved grooves are followed at the end of the Q-loop by a short 3_{10} helix, which seems to be conserved among the ECF transporters. This Q-helix has a conserved xPD/ExQφ (φ is a hydrophobic residue) motif, in which the conserved acidic residue and the conserved glutamine point to opposite sides of the helix. The latter points into the groove and is therefore thought to play a role in the interaction with transmembrane domains. The structures of the complete transporter also reveal a L-shaped EcfT consisting of eight α-helices, of which five are transmembrane helices (TM1-4 and TM8). Helix 5 lies at the cytoplasmic side of EcfT almost perpendicular to the transmembrane helices and helices 6 and 7 form two cytoplasmic coupling helices, which stick with their C-terminal ends into the grooves at the surface of EcfA' and EcfA, respectively. Together these coupling helices form a X-shaped bundle, which is stabilized by extensive hydrophobic interactions and hydrogen bonds between the conserved Glu174 in helix 6 and the

conserved Ser211 and Arg215 in helix 7. The C-terminal end of these helices is formed by a conserved XRX motif, in which the first X is usually an alanine and the second a glycine or serine (Neubauer et al. 2009). This signature motif participates in the interaction with the NBDs, and the interaction network is probably used to transfer conformational changes upon ATP binding and hydrolysis in the EcfAA' dimer to the transmembrane domains of the transporter.

In the transmembrane part of the transporter, the S-components bind in the cleft of the L-shaped EcfT, being separated from the EcfAA' dimer only by the coupling helices. Not TM1-3 which were predicted to interact with the ECF module, but TM1, TM2, and the C-terminal end of TM6 of all three S-components show a common surface, through which they interact with the coupling helices. The first coupling helix of EcfT mainly interacts with TM1 of the S-components via hydrophobic interactions and the AxxxA motif. The second coupling helix lies in a hydrophobic groove formed by TM1, TM2, and the C-terminal end of TM6 of the S-components. A similar groove is found in RibU, ThiT, and BioY. TM1, TM2, and the C-terminal end of TM6 contain several small hydrophobic residues that form an extensive hydrophobic interaction network with small hydrophobic residues in the coupling helix. This interaction network is important for the formation and stabilization of the whole transporter (Zhang et al. 2014). A second interaction site is formed by hydrophobic residues in the L3 and L5 loops of the S-components and hydrophobic residues in TM1, TM3, and TM4 in EcfT.

Mechanism of Transport

Type I Importers

The maltose transporter MalFGK$_2$E from *E. coli* is the best-studied Type I importer (Davidson et al. 2008; Oldham et al. 2007). In fact, a lot of what we know of full-length Type I ABC transporters comes from crystal structures of MalFGK$_2$-MBP, elucidated by the Davidson and Chen labs. Currently, structures of the maltose transporter in the inward-facing *apo*-state without the maltose-binding protein (MBP) (S_V in Fig. 7; Khare et al. 2009; Chen et al. 2013); in the outward-facing state with maltose, nucleotides, and MBP bound (S_{IV} in Fig. 7; Oldham et al. 2007; Oldham and Chen 2011b); and a pre-translocation state between the inward-facing and outward-facing state, with maltose and MBP bound and in the presence ($S_{III} \rightarrow S_{IV}$ in Fig. 7; Oldham and Chen 2011a) and absence of nucleotides ($S_{III} \rightarrow S_{IV}$ in Fig. 7; Oldham et al. 2013) are available. However, there is not yet a crystal structure of the nucleotide-bound inward-facing conformation (S_I–S_{III} in Fig. 7).

In growing cells, the levels of ATP (typically millimolar) are well above the K_D for binding of ATP to the NBDs and this primes the translocator for import (Fig. 7, $S_V \rightarrow S_I$). In S_I the translocator is in the nucleotide-bound inward-facing

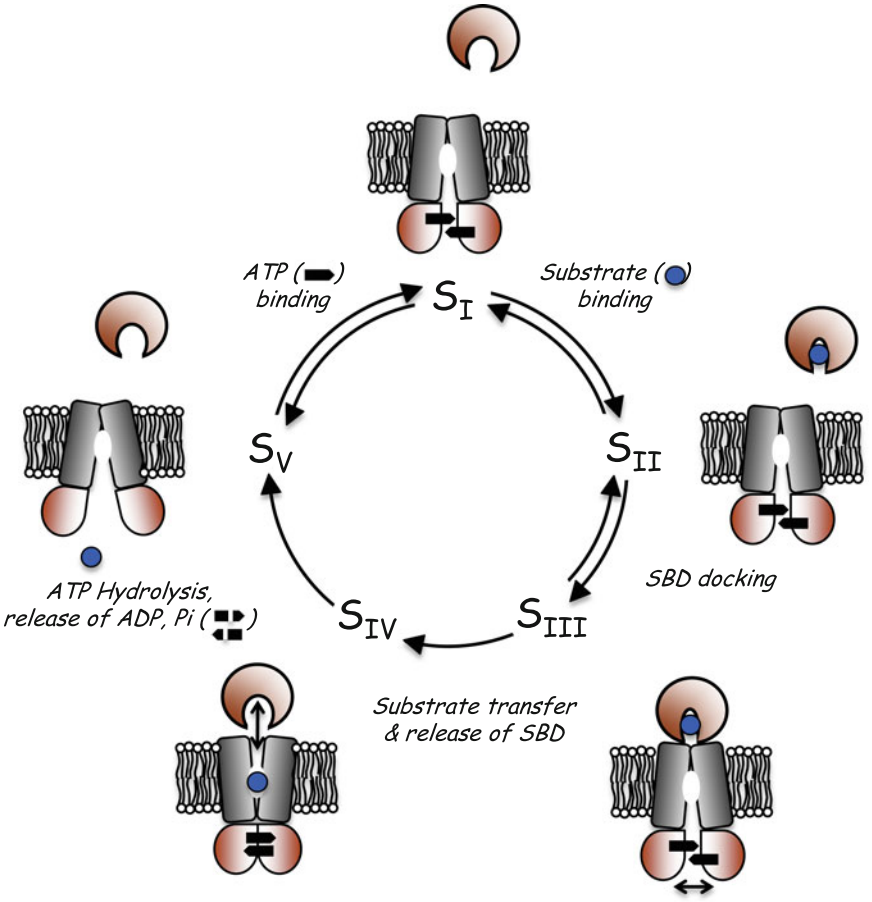

Fig. 7 Model of substrate translocation via a Type I ABC importer. After Gouridis et al. (2015)

conformation. Upon substrate capture via induced fit, the SBP changes conformation from open to closed (S_{II}) and, subsequently, docks onto the translocator (S_{III}). Through the binding of SBP in the CL state to the TMDs and Mg–ATP bound in the NBDs, the so-called pre-translocation state is formed (S_{III}), that is, the NBDs come closer together, forming a tipping for the access of the binding site in the TMDs. The binding energy of both ATP and SBP is used to transit the translocator from an inward to an outward-facing conformation ($S_{III} \rightarrow S_{IV}$), which allows the transfer of the substrate from the SBP to the TMD (S_{IV}). Subsequent hydrolysis of ATP and release of ADP and inorganic phosphate (Pi) complete the reaction cycle and release the substrate on the *trans* side of the membrane ($S_{IV} \rightarrow S_V$).

In another model of a Type I importer (Bao and Duong 2013), based on biochemical studies of the maltose transporter, the open state of the SBD interacts with the TMD in the outward-facing conformation, which then allows the binding of maltose. Upon subsequent hydrolysis of ATP, the transporter converts to the inward-facing conformation and releases the maltose into the cytoplasm. This model finds little support in the work on other Type I ABC importers, and the initiation of translocation through binding of SBP in the open state is controversial. In fact, the model of the Duong lab is based on studies in nanodiscs in which the coupling between maltose-MBP binding and ATP hydrolysis is relatively poor compared what others measure (Alvarez et al. 2010), and $MalFGK_2$-MBP may not have been in a fully native conformation.

An outstanding question is whether substrate recognition (selectivity) by ABC importers is counted for solely by the SBP or whether the TMD also contributes. For the maltose system there is data (Ferenci 1980; Ferenci et al. 1986; Treptow and Shuman 1985) suggesting that part of the selectivity originates from the TMD. For instance, mutants that function independent of the maltose-binding protein still transport no sugars other than maltodextrins (Treptow and Shuman 1985). In a recent structural study by the Chen lab (Oldham et al. 2013), it was shown that the transmembrane subunit MalF binds three glucosyl units from the non-reducing end of the sugar, suggesting that both MBP and the TMD contribute to the selectivity of the transporter. In accordance with these observations, the final step in translocation (Fig. 7, $S_{IV} \rightarrow S_V$) by GlnPQ is faster for asparagine than for glutamine (Gouridis et al. 2015). This implies that the two amino acids interact differently with the TMD and that additional selectivity to transport is provided beyond the step of substrate binding to the SBDs.

Another point of discussion is the stoichiometry of the transport reaction, which is technically challenging to determine. Many ABC transporters display poor coupling between ATP hydrolysis and translocation when purified and reconstituted in lipid vesicles, that is, significant amounts of ATP are hydrolyzed even in the absence of substrate. Another difficulty is that the substrate and ATP need to be present on opposite sides of the membrane, and the affinity of the TMD for the SBP is low (Prossnitz et al. 1989; Dean et al. 1992; Doeven et al. 2004). The Type I ABC importer OpuA transports glycine betaine and is gated by ionic strength. Like GlnPQ it has the SBD covalently linked (Fig. 3), which facilitates the membrane reconstitution and the ability to perform functional assays. Importantly, below threshold levels of ionic strength, there is hardly any ATP hydrolysis provided the lipid composition is "physiological" (>25 mol% of anionic lipids, 40–50 % non-bilayer lipids) (van der Heide et al. 2001; Biemans-Oldehinkel et al. 2006a). Under these conditions and upon activation of the transporter, the experimental ATP/glycine betaine ratio was 2.0 ± 0.5 (Patzlaff et al. 2003), suggesting a mechanistic stoichiometry of 2. At present, this is probably the best estimate of the ATP/substrate ratio for any ABC transporter.

A subset of ABC importers has additional domains fused to the NBDs, which serve to control the activity of the transporter. $MalFGK_2$-MBP from *E. coli* and OpuA from *Lactococcus lactis* are the best-studied systems in terms of regulation of

transport. The C-terminal domain of MalK of the maltose transporter is involved in carbon catabolite repression (Böhm et al. 2002), which determines the hierarchy of sugar utilization and is also known as an inducer exclusion mechanism. The C-terminal domain of MalK can interact with IIA^{glc}, a component of the bacterial phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS). IIA^{glc} can be in the unphosphorylated and phosphorylated states, depending on the availability of PTS sugars (Nelson and Postma 1984; Dean et al. 1991). In the unphosphorylated form, i.e., when a PTS sugar such as glucose is present, IIA^{glc} binds to MalK and thereby prevents uptake of maltose, the inducer of the maltose operon. It has been recently shown that IIA^{glc} stabilizes the inward-facing conformation of the maltose transporter by wedging between the NBD of one MalK and the regulatory domain of the opposite MalK (Chen et al. 2013). Herewith, the closure of the NBDs and thus the formation of the outward-facing conformation are prevented, which are key steps for ATP hydrolysis and maltose translocation. The inhibition is relieved when glucose is exhausted and IIA^{glc} becomes phosphorylated.

The activity of OpuA increases with the osmolality of the medium, which is signaled to the protein as an increase in the cytoplasmic electrolyte concentration (gating by ionic strength). Two cystathionine-β-synthase (CBS) domains fused in tandem (CBS module) to the C-terminus of the NBD and an anionic membrane surface are central to the gating of the transporter by ionic strength (Biemans-Oldehinkel et al. 2006b; Karasawa et al. 2013). CBS2 is involved in electrolyte sensing, whereas the CBS1 domain merely serves as linker between the nucleotide-binding domain (NBD) and the CBS2 domain (Karasawa et al. 2011). In one model the interaction of the CBS module with an anionic membrane surface locks the transporter in an inactive conformation. Increasing the intracellular ionic strength beyond threshold levels screens the electrostatic interactions of oppositely charged surfaces and activates the transporter. Alternatively, the ionic gating of OpuA could involve two like-charged surfaces, such as the anionic membrane and anionic protein residues, in which case a high ionic strength would promote their interaction. The verdict is not yet out but it is evident that screened electrostatic forces and an anionic membrane surface are intrinsic to the gating mechanism of OpuA.

For a number of Type I ABC transporters, it has been observed that *trans*-accumulated substrate inhibits the translocation. In *Listeria monocytogenes* and *Lactobacillus plantarum*, the transport of glycine betaine and carnitine is inhibited by pre-accumulated (*trans*) substrate at 0.1 M or above (Verheul et al. 1997; Glaasker et al. 1998). Upon raising the medium osmolality, the corresponding ABC transporters are rapidly activated through a diminished *trans*-inhibition. Once cellular osmostatic conditions are restored, the transporters are switched off. This so-called *trans*-inhibition serves as a control mechanism to prevent the accumulation of the compatible solutes glycine betaine and carnitine to too high levels and thereby prevents the turgor pressure from becoming too high. Although the physiological effect is the same, *trans*-inhibition is mechanistically different from the ionic strength gating observed in OpuA. The ABC transporter OpuA is not inhibited by *trans*-substrate up to at least 400 mM (Patzlaff et al. 2003). For two

Type I ABC transporters the structural basis for *trans*-inhibition has been elucidated, that are the methionine transporter MetN2I2 from *E. coli* and the molybdate transporter ModB2C2 from *Methanosarcina acetivorans*. In both cases, the NBD subunits have a C-terminal regulatory domain albeit with distinct folds (Gerber et al. 2008; Johnson et al. 2012). The MetN2I2 system is inhibited when cytoplasmic levels of methionine reach threshold values (Kadner 1977), and the binding of methionine to the C-terminal domain stabilizes the transporter in the inward-facing conformation, similar to what IIA^{glc} does to the maltose transporter. The ModB2C2 system is inhibited by the binding of molybdate or tungstate to its C-terminal regulatory domain.

In summary: In recent years, we have obtained enormous insight into the energetics and mechanism of transport and selectivity for ligands, as well as the gating of a subset of Type I importers. The regulation of ABC transporters is typically affected through one or more additional domains, linked to the NBD, which allow the transporter to be fully switched off under certain nutrient or osmotic conditions, even if substrate and ATP are available.

Type II Importers

Type I and Type II importers differ in their architecture of the SBP and TMD, but also mechanistically these transporters appear to operate differently (Davidson et al. 2008; Rees et al. 2009; Lewinson et al. 2010; Joseph et al. 2011). The mechanism of transport by a Type II importer presented in Fig. 8 is based on crystal structures of each of the states (Korkhov et al. 2014). The vitamin B_{12} transporter BtuCDF from *E. coli* is the best-studied Type II importer (Locher et al. 2002), and most of the states are based on this transporter; state S_{III} is based on the structure of $MolB_2C_2$ from *Haemophilus influenzae* (Pinkett et al. 2007). The structural analysis suggests two cytoplasmic and one periplasmic gate(s), depicted as vertical (red) and horizontal (gray) bars, respectively (Fig. 8). S_I represents the nucleotide-bound, outward-facing conformation. Ligand-bound SBP docks onto the translocator and this results in an occluded state (S_{II}), following the diffusion of the ligand from the SBP to the TMD. Next, ATP is hydrolyzed and inorganic phosphate and ADP are released, which opens the cytoplasmic gate II and the substrate diffuses into the cytoplasm (S_{III}). The $S_{III} \rightarrow S_{IV}$ transition is only possible after the substrate has been released, as in the case of BtuCDF the cavity in the TMD has become too small to accommodate vitamin B_{12}. Korkhov et al. (2014) speculate that S_{IV} may prevent the back-reaction or non-specific solute transport through the translocation pathway. Next, the SBP dissociates from the translocator and the TMDs rearrange to form an outward-facing conformation (S_V). Given the high intracellular concentrations of ATP, S_V is rapidly converted into S_I; the ATP binding closes the cytoplasmic gate II and the system is reset for another translocation cycle.

The BtuCDF system has a very high apparent ATP/substrate stoichiometry, i.e., of the order of 50 (Borths et al. 2005), which may reflect a high rate of ATP

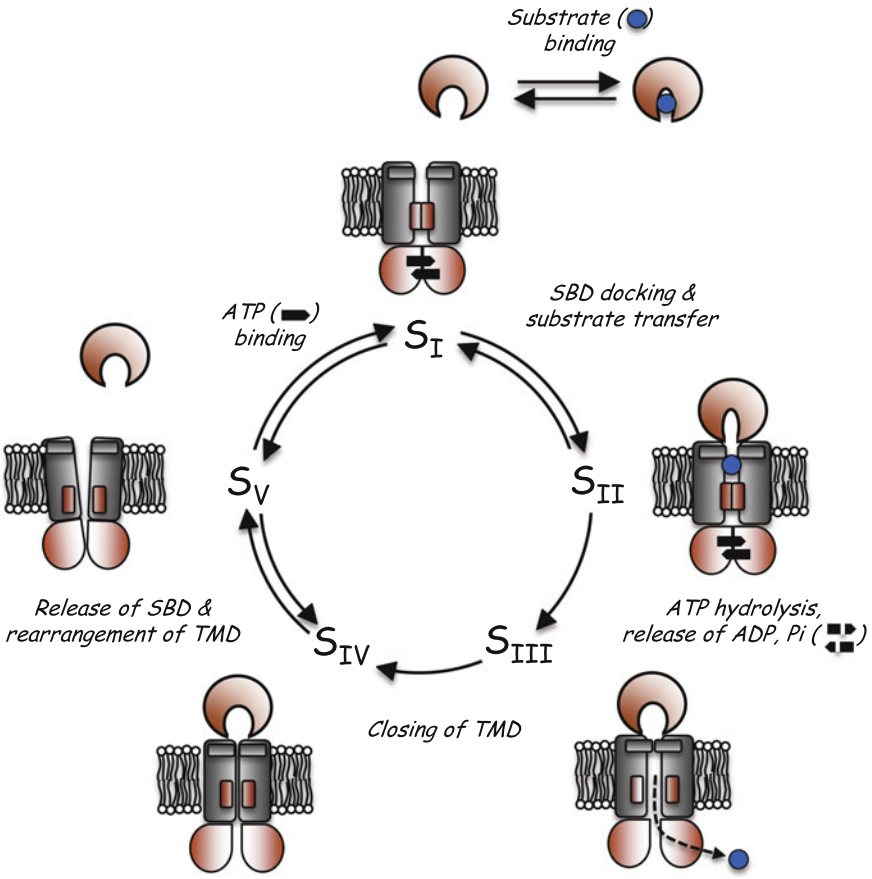

Fig. 8 Model of substrate translocation via Type II ABC importer. After Khorkhov et al. (2014)

hydrolysis of S_I or, as Korkhov et al. (2014) propose, S_{IV} to S_I-like transitions. This results in futile ATPase activity and a high apparent ATP/substrate stoichiometry; the mechanistic stoichiometry for full translocation is most likely 2. Contrary to the maltose transporter, there are no specific interactions between the substrate (vitamin B_{12}) and the TMD (BtuC subunits). The substrate pocket is very hydrophobic and forms a "Teflon-like" wall to prevent interactions with the hydrophilic substrate (Korkhov et al. 2012b). The NBDs of Type II importers discovered so far do not have extra domains and gating mechanisms (osmotic, catabolite, or product control) are not known.

In summary: Although Type I and Type II importers both employ extracytoplasmic SBPs or SBDs and operate by alternate access of the binding pockets in the TMDs, the mechanism of transport is very different. For instance, in the maltose transporter (Type I) the liganded SBP interacts with inward-facing TMD, whereas in the vitamin B_{12} transporter (Type II) the liganded SBP interacts with outward-facing

TMD. Accordingly, the subsequent steps are different for Type I and Type II importers.

Type III Importers

Apart from what has been revealed by the three crystal structures of the complete ECF FolT, ECF PantT, and ECF HmptT transporters from *L. brevis*, not much is known about the transport mechanism of the Type III importers. Unlike the Type I and Type II importers, the Type III importers contain a transmembrane domain that binds the substrate specifically without the help of a soluble SBP. At least 21 different S-component families are known, which are able to bind a wide range of small, chemically different substrates (Rodionov et al. 2006, 2009). Molecular dynamic simulation studies on the S-component ThiT for thiamin from *L. lactis* have shown that the six hydrophobic α-helices of the protein zig-zag through the lipid bilayer, with the connecting loops being located alternatively on the cytoplasmic and the extracellular side of it (Majsnerowska et al. 2013). In the four crystal structures of the substrate-bound S-components available, the substrate-binding site is located near the extracellular surface of the proteins (Zhang et al. 2010; Erkens et al. 2011; Berntsson et al. 2012; Yu et al. 2014). The site is formed by residues in the three C-terminal helices of the S-components together with residues in the L1 and L3 loops. These residues create specific, high-affinity binding sites with K_d values in the low nanomolar range (Erkens and Slotboom 2010; Zhang et al. 2010; Berntsson et al. 2012). The structure of the binding site of ThiT has been shown to be very robust, suggesting that a substantial input of energy, presumably in the form of ATP hydrolysis, is needed to accomplish conformational changes in the binding site to release its substrate (Swier et al. 2015). The N-terminal helices of the S-components are structurally more similar and provide interaction sites to interact with the EcfT domain. In the substrate-bound S-components, the substrate-binding site is closed by the L1 loop. However, in the structures of the complete transporters the L1 loop has moved away, opening the binding site to the cytoplasm (Wang et al. 2013; Xu et al. 2013; Zhang et al. 2014). This is in agreement with EPR studies performed on ThiT, which showed that the major difference between substrate-bound and substrate-free ThiT is a conformational change of the L1 loop (Majsnerowska et al. 2013).

In the structure of the complete transporters, the S-component adopts a highly unusual orientation, lying almost parallel in the membrane like it has toppled over from the orientation of the solitary S-components (S_I in Fig. 9) (Wang et al. 2013; Xu et al. 2013; Zhang et al. 2014). The S-component interacts with EcfT, but not with the NBDs, via two interaction sites with the interactions between TM1, TM2, and the C-terminal end of TM6 of the S-components and the coupling helices of EcfT forming the largest interaction interface. The two coupling helices create a X-shaped structure, of which the C-terminal ends stick into the grooves between the RecA-like domain and the helical domain at the surface of the EcfAA' dimer.

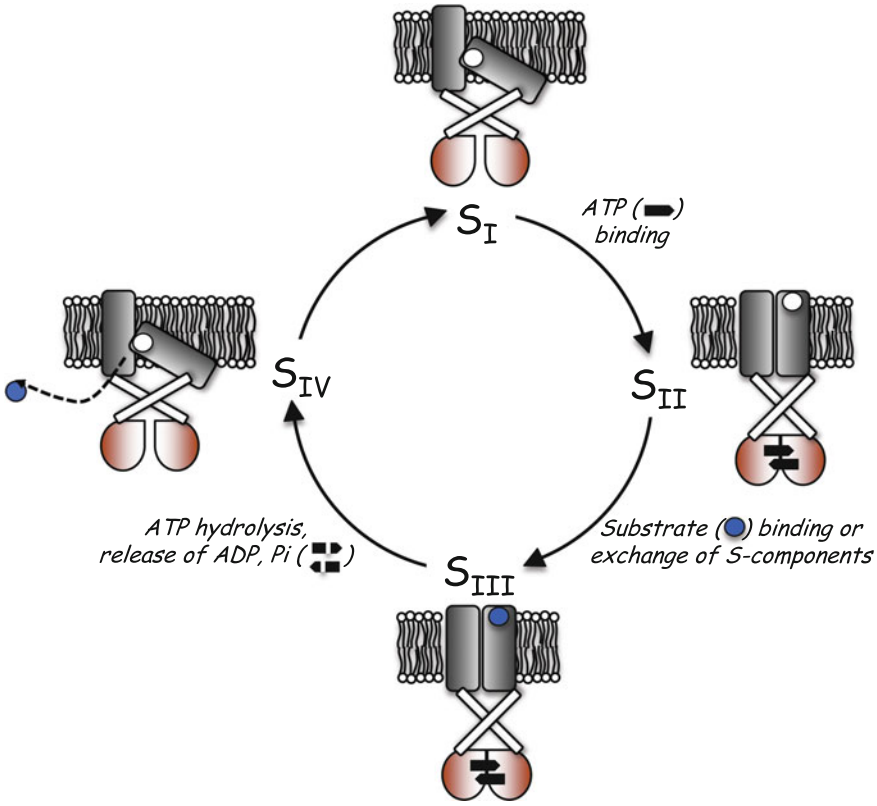

Fig. 9 Model of substrate translocation via a Type III ABC importer

Via an interaction network of electrostatic interactions and hydrogen bonds, these coupling helices are thought to transfer the conformational changes upon ATP binding and hydrolysis to the S-component. Like the NBDs in other ABC transporters, the EcfAA' dimer will undergo a tweezer-like closure upon ATP binding (S_{II} in Fig. 9) (Finkenwirth et al. 2015). This will probably push the C-terminal ends of the coupling helices together, creating a scissor-like conformation. The interactions between the S-component and EcfT are mainly hydrophobic (van der Waals), which allows the subunits to slide along each other. ATP binding might allow the S-component to rotate back to the up-right conformation in the membrane in order to capture its substrate on the extracytoplasmic side, while maintaining the interaction with EcfT (Finkenwirth et al. 2015). It is also possible that in this orientation, the interaction between the substrate-free S-component and EcfT is weakened and the two will dissociate, allowing another substrate-bound S-component to bind (S_{III} in Fig. 9) (Karpowich et al. 2015). Early research has shown that within the group II ECF transporters multiple S-components can compete for the same ECF module and that the affinity of the S-component for the

ECF module is dependent on the substrate concentration, with substrate-bound S-components binding stronger to EcfT than S-components in the absence of their substrate (Henderson et al. 1979). ATP hydrolysis and subsequent release of the hydrolysis products will then allow the coupling helices to move back to the open-scissor conformation and allow the S-component to topple over (S_{IV} in Fig. 9). The energy generated by ATP hydrolysis could be used to lower the binding affinity for the substrate, so it can be released into the cytoplasm. This mechanism would be new form of the moving-carrier mechanism, in which the S-component moves as a rigid body like in the elevator mechanism, but topples over to expose the substrate binding site to the cytoplasm, rather than making a translational movement as in the elevator mechanism.

A 1:1:1:1 stoichiometry was found in the crystal structures of the complete transporters and this stoichiometry has been confirmed by light scattering experiments performed on the group II ECF transporters from *L. lactis* (ter Beek et al. 2011). On the other hand, a 1:1:2:2 (A:A':T:S) stoichiometry was suggested for the group II riboflavin transporter from *Thermotoga maritima* (Karpowich and Wang 2013) and oligomeric EcfT and S-components were suggested for the group I biotin transporter from *Rhodobacter capsulatus* (Finkenwirth et al. 2010; Neubauer et al. 2011; Kirsch et al. 2012). The former result is difficult to reconcile with the crystal structures, but in the latter case it is possible that two or more complexes oligomerized in the membrane, and that the basic complex has 1:1:1:1 stoichiometry. Besides the debate on stoichiometry, rare solitary S-components have been found that seem to function independent of the ECF module, i.e., they are found in organisms that do not have the genes for the ECF module. An example is the biotin-specific S-component BioY, found in almost all Chlamydia strains, diverse proteobacterial and cyanobacterial species (Rodionov et al. 2009; Fisher et al. 2012; Finkerwirth et al. 2013). Furthermore, BioY and the cobalt-specific S-component CbiMN from *Rhodobacter capsulatus* may facilitate substrate entry into the cell in the absence of the ECF module (Hebbeln et al. 2007; Siche et al. 2010). Maybe these S-components topple over spontaneously in the membrane, a transition that would be energetically unfavourable but such rare events may suffice to allow the cell to take up small amounts of micronutrients. The S-components could also oligomerize and subsequently assist each other in the toppling in the membrane, or they could be assisted by unknown protein(s).

In conclusion: Type I and Type II ABC importers employ extramembranous substrate-binding proteins to capture their substrate and deliver the molecule to the translocator. Type III ABC importers use an integral membrane protein (S-component) for the initial binding of substrate; transport is thought to take place by reorientation of the S-component in the membrane. Whereas full structures of Type I and Type II ABC importers with/without SBPs are available, the translocation mechanism by Type III importers awaits further biochemical and structural analysis.

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Bacterial ABC Multidrug Exporters: From Shared Proteins Motifs and Features to Diversity in Molecular Mechanisms

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Abstract Bacterial ATP-binding cassette (ABC) exporters embrace an enormous range of biological processes. They can mediate the efflux of a wide variety of substrates ranging from small inorganic ions, drugs, and antibiotics to large protein toxins and other macromolecules. They can also act as mediators and regulators in transmembrane signaling processes perhaps without mediating any direct transport reaction. This diversity in function of ABC exporters raises questions about their structure, how conformational changes are coupled to activity, and how we can use this information to inhibit, activate, or bypass physiological functions in drug-based strategies. When the first ABC transporters were discovered, now 40 years ago, it was noted by sequence comparisons that many of them shared a similar domain organization. But exactly how these domains cooperate in mediating transport activity was unknown. A wealth of biochemical studies and crystal structures of nucleotide-binding domains (NBDs), and subsequently of full-length ABC exporters, suggests that the general mechanism is based on metabolic energy-dependent alternating access of substrate-binding pocket(s) to either side of the phospholipid bilayer, but that there is diversity in the detailed molecular mechanisms that are being employed. This chapter provides an overview of the structural and mechanistic intricacies that have surfaced over the past years, and the challenges in further studies on these amazing transport proteins.

Introduction

ABC transporters have been studied since the 1970s and 1980s, when research focused on bacterial systems that mediate the import of solutes such as histidine, maltose, or peptides into the cell (Ames et al. 1977; Bavoil et al. 1980; Higgins

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et al. 1982; Gilson et al. 1982; Hiles and Higgins 1986), or the export of toxins, antimicrobial peptides, and antibiotics (Felmlee et al. 1985; Vogel et al. 1988; Gilson et al. 1990; Guilfoile and Hutchinson 1991). This coincided with the discovery of the highly conserved ATP-binding cassette in these transport proteins, and the designation of "ABC transporter" (Higgins et al. 1986). Research also focused on ABC exporters in mammals as early as 1976, when P-glycoprotein (ABCB1) was recognized as a surface glycoprotein that was overexpressed in drug-resistant Chinese hamster ovary cells (Juliano and Ling 1976). Subsequent cloning and sequencing of human ABCB1 cDNA from a multidrug-resistant cell line in 1986 allowed the assignment of ABCB1 to the same protein family as the bacterial proteins (Riordan et al. 1985; Chen et al. 1986; Ueda et al. 1987). With the discoveries and gene sequencing of many other important mammalian ABC proteins including the chloride channel CFTR (Riordan et al. 1989), multidrug resistance-associated proteins MRP1 (Cole et al. 1992) and MRP2 (Buchler et al. 1996), and transporters for antigen processing TAP1/2 (Spies et al. 1990), ABC transporters rapidly expanded into a superfamily.

Many mammalian ABC exporters show evidence of a relationship to bacterial ABC half-transporters that dimerize for function. The gene structures of proteins such as ABCB1 and CFTR point to a past internal gene duplication. Phylogenetic analyses indicate that these duplications occurred independently, while a tandem gene duplication occurred in the case of the TAP family (Hughes 1994; Dean and Allikmets 1995). ABCB1 is closely related to the hemolysin A-secreting HlyB transporter (Holland et al. 1991) and lipid A transporter MsbA (Karow and Georgopoulos 1993) in Gram-negative bacteria and multidrug transporter LmrA and orthologs in Gram-positive bacteria (van Veen et al. 1996; van Veen and Konings 1997). In accordance with the endosymbiont theory, one hypothesis to explain this observation is that eukaryotic ABCB1 and TAP genes are descended from a mitochondrial gene or genes that were subsequently translocated to the nuclear genome. Indeed, subsequent studies demonstrated that LmrA and MsbA can interact with similar substrates including lipid A, and that they exhibit a similar selectivity for cytotoxic drugs and modulators as ABCB1. Therefore, these proteins share functional and structural properties (van Veen et al. 1998; Reuter et al. 2003; Woebking et al. 2005). As this conclusion was drawn well before the entry of structural biology into the field, it is useful to emphasize that this conclusion is also supported by X-ray crystal structures of MsbA (Ward et al. 2007) and ABCB1 (Aller et al. 2009; Jin et al. 2012).

Genome sequencing and transcriptome analyses on a variety of micro-organisms allowed the expansion of the microbial branch of the ABC superfamily (Linton and Higgins 1998; Quentin et al. 1999; Moussatova et al. 2008), and underscored the importance of some of these new bacterial members in conferring antibiotic resistance on cells. In 2006, Piddock and coworkers described the overexpression of two genes encoding putative ABC half-transporters, *patA* (SP2075) and *patB* (SP2073), in multidrug resistant *Streptococcus pneumoniae* (Marrer et al. 2006a, b), which exhibited enhanced efflux of various drugs. In parallel, Robertson et al. (2005) inactivated 13 genes encoding putative efflux pumps in *S. pneumoniae*.

Inactivation of SP2075 or SP2073 gave rise to hypersusceptibility to multiple drugs including antibiotics. Subsequent work suggested that these proteins, renamed to PatA and PatB, confer innate multidrug resistance on *S. pneumoniae* by forming a heterodimeric multidrug pump PatAB (Garvey and Piddock 2008; Boncoeur et al. 2012). Other observations also suggest that overproduction of *patA* and *patB* is a clinically relevant mechanism of resistance in fluoroquinolone-resistant clinical isolates of *S. pneumonia* (Garvey et al. 2011). Orthologs of PatAB exist in other Gram-positive organisms such as LmrCD from *Lactococcus lactis* (Zaidi et al. 2008) and BmrCD from *Bacillus subtilis* (Galian et al. 2011), and similar to PatAB, endogenous expression of these transporters was found to have strong effects on the intrinsic resistance of the respective organisms to cytotoxic agents.

Thus, research over the past 40 years has identified bacterial ABC multidrug exporters as exciting proteins that can serve as accessible models for their mammalian counterparts, but that are also highly interesting in their own right because of important physiological roles of such systems in the transport of a wide variety of substrates including antibiotics, (toxic) ions, lipids, peptides, and many others in bacteria.

Domain Organization and Interactions

In the pre-crystal structure era of the 1990s, protein sequence comparisons and hydropathy analyses suggested that bacterial ABC multidrug exporters typically consist of four domains, two membrane domains (MDs) and two nucleotide-binding domains (NBDs). The MDs are thought to contain the translocation pathway for the substrate, and as there is variety in transport substrates, there is less sequence conservation in MDs compared to NBDs. The NBDs are the engine of the transporter and contain features that allow binding and hydrolysis of ATP: Walker A and B motifs, ABC signature sequence (or C motif), conserved residues in specific areas such the A-, D-, H-, and Q-loops, and conserved sequences in loop regions that communicate conformational changes from the NBDs to the MDs (Kerr 2002; Hanekop et al. 2006; Zaitseva et al. 2006). In many of these exporters, one MD and one NBD are fused into a half-transporter. In a variety of genetic and biochemical experiments it was demonstrated for LmrA and BmrA that these half-transporters dimerize (van Veen et al. 2000; Ravaud et al. 2006) to form the same functional unit as the monomers of mammalian ABC exporters in which the domains are fused into a single polypeptide (Loo and Clarke 1996; Taylor et al. 2001).

With the arrival of the first crystal structures of bacterial ABC half-transporters Sav1866 from *Staphylococcus aureus* (Dawson and Locher 2006, 2007) and MsbA from Gram-negative bacteria (Ward et al. 2007), many details regarding the domain organization were elucidated. The putative membrane-spanning segments in the MDs were indeed found to be α-helical, and the interactions between the two half-transporters were supported by the exchange of helical hairpins between the

two MDs. Furthermore, nucleotide binding at the NBDs was found to be associated with domain dimerization, consistent with earlier observations of fluorescence energy transfer and cysteine cross-linking between the NBDs in ABCB1 (Loo and Clarke 2000a; Urbatsch et al. 2001; Qu and Sharom 2001) and the first crystal structures of the ATP-bound sandwich dimers of isolated ABC–NBDs (Smith et al. 2002; Yuan et al. 2001). These structural insights and accompanying biochemical evidence have led to a general model in which ATP-dependent conformational changes in the NBDs drive rearrangements of the transmembrane helices (TMHs) to enforce the link between transport and ATP hydrolysis, referred to as conformational coupling, to achieve a transport mechanism often referred to as 'alternating access'.

Alternating Access Mechanisms

Already in the early days of transporter research (Mitchell 1957; Jardetzky 1966), these proteins have been viewed as undergoing conformational changes in which a central binding site is exposed alternately to the inside or the outside of the cell, but never simultaneously to both sides. Groundbreaking studies on the proton-lactose co-transporter LacY and other secondary-active solute transporters indeed identified the substrate-binding site in a pocket at similar distance from either side of the membrane and near the molecular twofold axis of transporters. The structural change between inward- and outward-facing conformations in LacY involves rotation between the N- and C-terminal domains around the substrate-binding site, thereby allowing the binding site alternating accessibility to each side of the membrane (Abramson et al. 2003; Law et al. 2008; Gouaux 2009).

The first biochemical evidence for a change in accessibility of drug-binding sites in ABC multidrug exporters in response to nucleotide binding came from equilibrium drug-binding studies on human ABCB1 (Martin et al. 2000b) and the breast cancer resistance protein ABCG2 (McDevitt et al. 2008), which suggested that the binding of ATP, rather than its hydrolysis, causes the initial conformational shift in the drug-binding site to a low-affinity state. Studies in parallel on bacterial LmrA (van Veen et al. 2000) suggested that high- and low-affinity substrate-binding sites are accessible on the internal and external membrane surface, respectively, with occlusion of the high-affinity site in an ATP-bound state. LmrA was the first ABC extrusion system to be functionally reconstituted in proteoliposomes in a transport-active form, enabling detailed analysis of its mechanism of action and paving the way for the study of many more putative bacterial ABC transporters (Margolles et al. 1999). This work was followed by extensive pharmacological characterizations of drug-binding sites in LmrA and ABCB1 using transport and equilibrium drug-binding assays. These studies revealed the presence of communicating transport-active sites for substrates, for example for vinblastine and Hoechst 33342 in LmrA (van Veen et al. 2000) and vinblastine, Hoechst 33342, and rhodamine 123 in ABCB1 (Shapiro and Ling 1997; Martin et al. 2000a;

Lugo and Sharom 2005). In spite of these significant advances, the lack of structural information on ABC transporters limited molecular interpretations of these findings; ABC exporters remained a black box for many of the following years.

Alternating Access in a Structural Biology Context

The first structures for ABC exporters were published for Sav1866 from *S. aureus* (Dawson and Locher 2006, 2007) and MsbA from Gram-negative bacteria (Ward et al. 2007) (Table 1). These structures were named "outward-facing" (Sav1866, MsbA) and "inward-facing" (MsbA) in accordance with the accessibility to the outer membrane leaflet or inner membrane leaflet, respectively, of the central cavity that is enclosed by the two MDs of these homodimeric transporters. Related conformations were also observed in later crystal structures of ABC exporters from prokaryotic and eukaryotic organisms (Table 1). Biochemical and biophysical analyses of various MsbA conformations by EPR/DEER-based distance measurements in nitroxide spin-labeled MsbA in proteoliposomes (Zou et al. 2009; Zou and McHaourab 2009), cryo-electron microscopy of purified MsbA (Ward et al. 2009), and disulfide cross-linking of MsbA in membrane vesicles (Doshi et al. 2010, 2013) suggest that release of the hydrolysis products ADP and phosphate promotes an inward-facing conformation that binds the substrate, whereas the binding of ATP promotes the dimerization of NBDs in an outward-facing conformation from which substrate dissociates.

The Sav1866 and MsbA crystal structures revealed significant information about transmembrane helix packing and domain interactions. Firstly, in addition to NBD–NBD interactions, referred to earlier, these structures also provided invaluable insights into the communication between NBDs and MDs (Dawson and Locher 2006, 2007), and were found to be useful as templates for studies on related systems such as ABCB1 (Zolnerciks et al. 2007). Secondly, comparisons of the protein structures reveal the swapping of helical hairpins formed by TMH 1 and 2 between the MDs of the half-transporters in the outward-facing conformation, and TMH 4 and 5 in the inward-facing conformation. Hence, the conformational transitions between the inward-facing and outward-facing states require major structural rearrangements in the MDs, and a different intertwining of the half-transporters in both states. The transition between these two states might proceed via an intermediate state without this intertwining. This state was recently captured in a crystal structure for the antibacterial peptide transporter McjD from *E. coli*, which lacks intertwining and shows a well-defined binding cavity that is closed to all sides (Choudhury et al. 2014), and was also suggested for MsbA based on biochemical evidence (Doshi and van Veen 2013). When taken together, the current structures of Sav1866, MsbA, and McjD indicate that these ABC exporters adopt an inward-facing conformation in the absence of nucleotide or when bound to ADP, and that they advance via an ATP-bound occluded transition state into the ATP-bound outward-facing conformation. ATP hydrolysis finally enables dissociation of the

Table 1 Crystal structures of polyspecific ABC exporters

Protein	Organism	Conformation	Nucleotide	Inhibitor	PDB	Resolution (Å)	References
Sav1866	*Staphylococcus aureus*	Outward	ADP[b]		2HYD	3.00	Dawson and Locher (2006)
Sav1866	*Staphylococcus aureus*	Outward	AMP-PNP		2ONJ	3.40	Dawson and Locher (2007)
MsbA	*Escherichia coli*	Inward			3B5W	5.30	Ward et al. (2007)
MsbA	*Vibrio cholerae*	Inward			3B5X	5.50	Ward et al. (2007)
MsbA	*Salmonella typhimurium*	Outward	ADP-PNP		3B5Y	4.50	Ward et al. (2007)
MsbA	*Salmonella typhimurium*	Outward	AMP-OV		3B5Z	4.20	Ward et al. (2007)
MsbA	*Salmonella typhimurium*	Outward	AMP-PNP		3B60	3.70	Ward et al. (2007)
McjD	*Escherichia coli*	Occluded	AMP-PNP		4PL0	2.70	Choudhury et al. (2014)
ABCB1a	Mouse	Inward			3G5U	3.80	Aller et al. (2009)
ABCB1a	Mouse	Inward		QZ59-RRR	3G60	4.40	Aller et al. (2009)
ABCB1a	Mouse	Inward		2 × QZ59-SSS	3G61	4.35	Aller et al. (2009)
CeABCB1	*Caenorhabditis elegans*	Inward			4F4C	3.40	Jin et al. (2012)
CmABCB1	*Cyanidioschyzon merolae*	Inward			3WMF	2.60	Kodan et al. (2014)
CmABCB1	*Cyanidioschyzon merolae*	Inward		aCAP	3WMG	2.40	Kodan et al. (2014)
ABCB10	Human mitochondria	Inward	AMP-PCP		4AYX	2.90	Shintre et al. (2013)
Atm1	Yeast mitochondria	Inward			4MYC	3.06	Srinivasan et al. (2014)
NaAtm1	*Novosphingobium aromaticivorans* mitochondria	Inward			4MRN	2.50	Lee et al. (2014)
TM287/288	*Thermotoga maritima*	Inward			4Q4H	2.53	Hohl et al. (2014)
TM287/288	*Thermotoga maritima*	Inward	AMP-PNP		4Q4A	2.60	Hohl et al. (2014)

[a]Conformation refers to the inward- or outward-facing orientations of the cavity formed by the MDs

[b]*ADP* Adenosine-di-phosphate, *AMP-PNP* Adenosine 5′-(β,γ-imido)triphosphate, *ADP-OV* Adenosine-di-phosphate-orthovanadate, *QZ59-RRR* cyclic-tris-(R)-valineselenazole, *QZ59-SSS* cyclic-tris-(S)-valineselenazol, *aCAP* anti-CmABCB1 peptide

NBD dimer and resets the transporters to the inward-facing conformation. It is not clear yet whether the ATP-induced dimerization of the NBDs alone [also referred to as 'ATP switch' model (Higgins and Linton 2004)] is sufficient to cause the transition from the inward-facing state to the outward-facing state. As formulated in the 'occlusion-induced switch' model (reviewed by Seeger and van Veen 2009) this transition might require the occlusion or tight binding of a nucleotide following NBD dimerization. Furthermore, the possibility exists that conformational transitions in ABC exporters are affected by transmembrane electrochemical ion gradients (Venter et al. 2003; Choudhury et al. 2014). Thirdly, the notion that the central cavity at the MD–MD interface can indeed act as a substrate-binding pocket during alternating access was supported by a structure of mouse ABCB1a in complex with peptide inhibitors (Aller et al. 2009), and by superimposition of known change-in-drug-specificity mutations in ABCB1 (Loo and Clarke 2000b, 2008) and MsbA (Woebking et al. 2008) on the structures of these proteins (Gutmann et al. 2010). The studies on ABCB1 suggest that structurally dissimilar substrates interact with sets of polar and aromatic side chains in a flexible binding chamber (Stockner et al. 2009) in a fashion that is analogous to substrate binding in protein crystals of multidrug-binding transcriptional regulators (Zheleznova et al. 1999; Schumacher et al. 2001) and the resistance-nodulation-cell division (RND) transporter AcrB (Murakami et al. 2006; Seeger et al. 2006). Alternating access of this binding chamber in the MDs is achieved through small-scale tilting and rotation of several individual TMHs with respect to each other (Omote and Al-Shawi 2006; Gutmann et al. 2010; Crowley et al. 2010). More recently, alternating access of the central binding chamber in response to nucleotide binding was also shown for ABCB1 in an EPR approach (van Wonderen et al. 2014).

The notion that ATP binding drives the drug extrusion step (the reorientation of the drug-binding chamber from the cell's interior to the external face of the plasma membrane) whereas ATP hydrolysis and release of ADP and Pi allow reorientation of the chamber back to the interior, shows parallels with the catalytic mechanism proposed for bacterial ABC importers such as the maltose importer MalFGK2 and the vitamin B12 importer BtuCD (Davidson 2002; Chen et al. 2003; Dawson et al. 2007; Korkhov et al. 2012), and is also reminiscent of the earlier mechanisms proposed for secondary-active transporters. It provided a sense of existence of a unifying theory for the transport mechanism of ABC exporters, which turned into a narrow template for some.

Diversity in Mechanisms

While bacterial ABC exporters such as LmrA, and eukaryotic ABC exporters such as ABCB1 require two functionally active ATPase sites for transport (van Veen et al. 2000; Hrycyna et al. 1998), it is interesting to note that others (referred to as heterodimeric ABC exporters) contain two asymmetric ATP sites. Bacterial LmrCD, BmrCD, and T287/288 (Zaidi et al. 2008; Galian et al. 2011; Hohl et al. 2012;

Mishra et al. 2014), yeast Pdr5 (Ernst et al. 2010), mammalian ABCB2/3 (also referred to as TAP1/2) (Vos et al. 2000; Seyffer and Tampe 2014), and many mammalian ABCC proteins (including the multidrug resistance-associated proteins MRP1 and MRP2) (Hipfner et al. 1999; Borst and Elferink 2002; Gottesman et al. 2002) are examples of the latter. One of these ATP sites is formed exclusively by consensus residues and operates as an ATPase unit, while the other site contains non-consensus substitutions in one or more motifs, and is referred to as the degenerate site. The non-consensus substitutions in the Walker B, H-loop, and signature motif within the degenerate ATP site inhibit ATPase activity and affect NBD dimer stability (Hou et al. 2002; Perria et al. 2006; Procko et al. 2006). The latter is also affected in the D-loop region of the degenerate site in TM287/288 (Hohl et al. 2014). The functional role of ATP binding and closed dimer formation in heterodimeric ABC proteins was first illustrated by studies on CFTR, where instead of mediating transport, the ATP-binding-dependent NBD dimerization opens a chloride channel, whereas hydrolysis of ATP at the consensus site closes the channel (Vergani et al. 2005). Although the basic reaction steps at the NBDs (ATP binding, ATP hydrolysis, ADP/Pi release) are shared between conventional ABC exporters and heterodimeric ABC exporters, the number, order, and kinetics of these steps most likely differ, thus giving rise to a diversity of mechanisms.

Mechanisms of transport might even be diverse within classes of ABC exporters. Although many conventional ABC export systems possess two functional ATPase sites, diversity might arise depending on whether two ATP molecules are hydrolyzed simultaneously or sequentially within one transport cycle, or hydrolyzed in an alternating fashion (van Veen et al. 2000; Jones and George 2013). The latter mechanism could be similar to that of heterodimeric ABC exporters within one transport cycle, but different from this class, the role of the ATP sites in ATP hydrolysis or exclusive ATP binding would swap in sequential transport cycles, thus making the requirement for two functional ATPase sites compulsory. Most of these mechanisms will involve an asymmetric stage or stages in terms nucleotide binding at some point of catalysis, for example with ADP binding to one site and ATP binding to the other, which adds to the complexity of analyses and correct assignment of mechanisms with current experimental techniques.

The degree of proximity of the two NBDs in structures of intact ABC exporters (Table 1) suggests that the structural differences between the open and closed dimers might vary from more subtle to complete dimer dissociation. Catalytic mechanisms might differ between transporters depending on the extent of cooperativity between the nucleotide-binding pockets, on the oligomeric assembly of which the ABC half-transporter is part [e.g., in stand-alone MsbA dimer versus multicomponent transporters such as MacA-MacB-TolC (Zgurskaya 2009; Hinchliffe et al. 2013)], and on signals arising from substrate binding in the binding pocket. As different ABC exporters translocate substrates with different physico-chemical properties, ranging from small inorganic ions, medium-sized antimicrobial agents and polysulfides, large lipids and polysaccharides, to very large polypeptides (see Table 1 and Higgins (1992) for overview), and combinations thereof (Velamakanni et al. 2009), whereas others such bacterial FtsEX

(de Leeuw et al. 1999) and mammalian SUR (Burke et al. 2008) act as mediators in transmembrane signaling processes perhaps without mediating any direct transport reaction, diversity in mechanisms will arise from this enormous diversity in substrates and physiological roles.

Summary

The past years of research on ABC exporters in bacteria and other organisms have provided important insights into their clinical relevance, and have revealed many details about the biochemistry, pharmacology, and structural biology of these proteins. These advancements now raise the appreciation that although ABC exporters share typical protein motifs and features, they have evolved in different directions, giving rise to a diversity in mechanisms that underlies the variety in physiological functions. It is expected that the increased knowledge will facilitate our ability to inhibit, activate, or bypass ABC exporter activities in drug-based strategies. The opportunities for further research are therefore truly dazzling.

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Export of Staphylococcal Toxins by a Conserved ABC Transporter

Seth W. Dickey, Som S. Chatterjee and Michael Otto

Abstract Staphylococci are important human pathogens. Several of these, first and foremost *Staphylococcus aureus*, rely on the secretion of phenol-soluble modulins (PSMs) to establish infections in a variety of disease types. PSMs are amphipathic, α-helical peptides with broad sequence diversity and a variety of functions in the infectious and non-infectious lifestyles of staphylococci. For example, many PSMs are strongly cytolytic, and most of them have pro-inflammatory capacities. Furthermore, PSMs help structure biofilms and promote the dissemination of biofilm-associated infection throughout the body. PSMs are secreted non-canonically by a recently discovered and dedicated ABC transport system named Pmt. Consisting of two membrane-spanning and two ATPase proteins, Pmt is conserved among all staphylococci. This review describes the discovery, features, and putative mechanism of Pmt. Furthermore, it discusses the potential of using the Pmt system as a single target to abolish secretion of all PSMs for virulence-oriented anti-staphylococcal therapy.

Keywords *Staphylococcus aureus* · ABC transporter · Virulence · Phenol-soluble modulins

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Staphylococcal Toxins

Staphylococcus aureus is a premier global pathogen, responsible for hospital- and community-associated infections that cause considerable morbidity and thousands of deaths in the U.S. alone (Lowy 1998; Klevens et al. 2007). Antibiotic resistance, in particular to methicillin, severely complicates treatments of many *S. aureus* infections (Lowy 2003). *S. aureus* often causes moderately severe to severe skin and lung infections, endocarditis, and osteomyelitis, as well as specific disease types such as staphylococcal toxic shock syndrome (TSS) or scalded skin syndrome (Lowy 1998). The virulence potential of *S. aureus* is mostly due to a large variety of secreted toxins, many of which are only produced by specific strains, as they are encoded on mobile genetic elements (Otto 2014). Examples are several leukotoxins such as the Panton–Valentine leukocidin (PVL) or the toxic shock syndrome toxin (TSST), which causes TSS. In contrast, other toxin genes constitute part of the core genome or are found on genetic elements common to virtually all *S. aureus* isolates. These include α-toxin, a key toxin of *S. aureus* that is involved in causing a variety of disease manifestations, and the phenol-soluble modulins (PSMs) (Berube and Bubeck Wardenburg 2013; Cheung et al. 2014).

Coagulase-negative staphylococci (CoNS), with *Staphylococcus epidermidis* being the best-studied species, are opportunistic pathogens that cause disease usually only in predisposed patients (Otto 2004, 2009). Often these are biofilm-associated infections introduced during the insertion of indwelling medical devices (Otto 2008). These species do not produce a large variety of toxins, their repertoire virtually being limited to the production of members of the PSM family (Otto 2004, 2009).

Phenol-Soluble Modulins

PSMs are amphipathic, α-helical peptides with pronounced surfactant-like properties (Cheung et al. 2014; Peschel and Otto 2013). They can be classified in two subfamilies: the α-type PSMs are ∼20–25 amino acids in length with the entire peptide forming an amphipathic α-helix. In *S. aureus*, these include the δ-toxin, PSM α1, PSM α2, PSM α3, and PSM α4. The β-type subfamily comprises peptides with a length of ∼44–45 amino acids, with the α-helical part found in the C-terminal region. In *S. aureus*, these comprise the PSMβ1 and PSMβ2 peptides (Wang et al. 2007). Some *S. aureus* strains also produce the α-type PSM-mec, which—in contrast to the other, core genome-encoded PSMs—is found on specific staphylococcal cassette chromosome (SCC) *mec* elements that carry methicillin resistance (Queck et al. 2009; Hiramatsu et al. 2001). Most other staphylococcal species also produce PSM peptides, but in addition to *S. aureus* (Wang et al. 2007), have only been completely and systematically analyzed on the gene and protein level in *S. epidermidis* (Yao et al. 2005; Vuong et al. 2004; Mehlin et al. 1999). β-type PSMs may be found using similarity searches; and many previously discovered amphipathic peptides are now recognized to belong to the β-type PSM family (such as the gonococcal growth inhibitor peptide of *Staphylococcus*

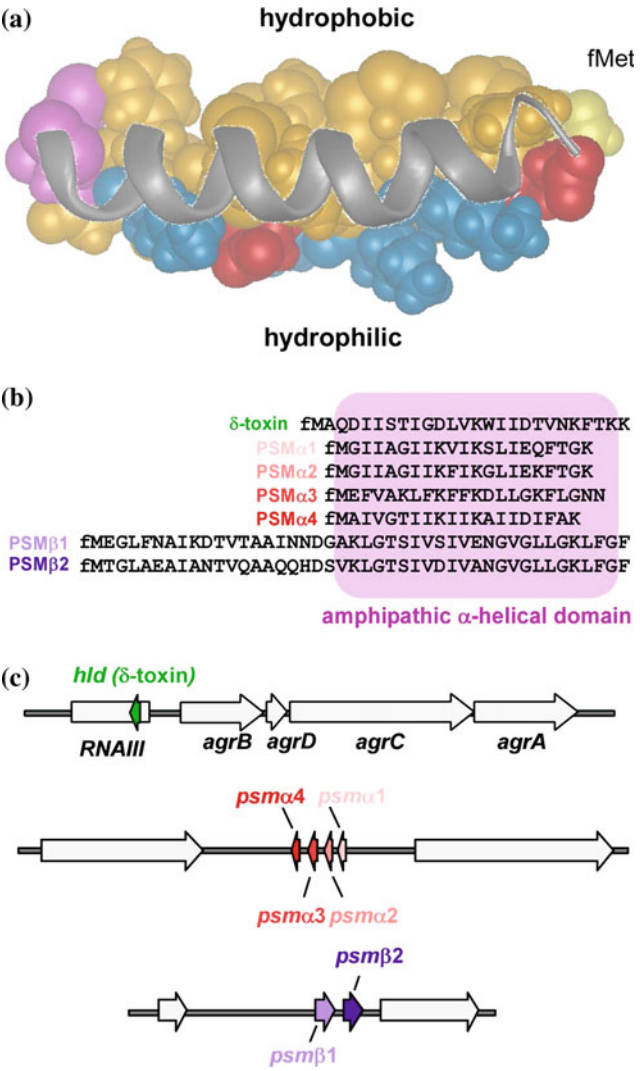

Fig. 1 Phenol-soluble modulins (PSMs). **a** PSMs adopt amphipathic, α-helices. PSMα3 modeled after the available NMR structure of δ-toxin is shown as example. Hydrophobic amino acids are in *brown*; positively and negatively charged amino acids in *red* and *blue*, respectively. The two C-terminal asparagine residues of PSMα3 (important for receptor interaction) are *in pink*. The N-terminal N-formyl methionine is in *yellow*. **b** Amino acid sequences of *S. aureus* PSMs. **c** Genetic loci encoding PSMs in *S. aureus*

haemolyticus and the slush peptides of *Staphylococcus lugdunensis*) (Cheung et al. 2014). In contrast, due to their short length and limited similarity on the gene and protein level, the elucidation of α-type PSM peptides and their genes requires purification and N-terminal sequencing (Wang et al. 2007; Joo and Otto 2014) (Fig. 1).

Research over the recent years has shown that PSMs are preeminent virulence factors contributing to a series of staphylococcal diseases (Wang et al. 2007, 2011; Kobayashi et al. 2011; Cassat et al. 2013; Rasigade et al. 2013; Nakamura et al. 2013; Periasamy et al. 2012), with increasing evidence suggesting that specific PSM subtypes are associated with distinct disease phenotypes. Many α-type PSMs (mostly *S. aureus* PSM α3) are strongly cytolytic, causing lysis of a variety of cell types including red and white blood cells, and have a strong impact on abscess formation and sepsis (Wang et al. 2007; Cheung et al. 2010, 2012). Cytolytic potential is believed to be mainly due to the pronounced amphipathy and α-helicity of PSMs (Laabei et al. 1838). In *S. aureus*, α-type PSMs can cause lysis of human neutrophils and non-professional phagocytes after ingestion, which represents an extremely efficient immune-evasion mechanism of *S. aureus* (Surewaard et al. 2013; Grosz et al. 2014). The δ-toxin of *S. aureus* has recently been recognized as a key factor contributing to the development of atopic dermatitis, working by facilitating mast cell degranulation (Nakamura et al. 2013). Interestingly, no other PSM was found to promote degranulation. The role of the β-type PSMs in disease is not yet entirely clear, but they may have a specific role during biofilm-associated infection, where they cause systemic dissemination of infection acting as biofilm-dispersing agents (Wang et al. 2011; Periasamy et al. 2012). However, all PSMs appear to be involved in that phenotype to some extent. In contrast to *S. aureus*, the role of PSMs in disease caused by CoNS is not yet well analyzed in vivo (except for the contribution of *S. epidermidis* β-type PSMs to biofilm structuring and dissemination of biofilm infection) (Otto 2009; Wang et al. 2011).

In addition to their cytolytic and biofilm-structuring properties, which are believed to be due to receptor-independent disintegration of membrane structures and the surfactant properties of PSMs, respectively (Laabei et al. 1838; Duong et al. 2012), PSMs have pro-inflammatory capacities that are mediated by the activation of the formyl peptide receptor 2 (FPR2) (Kretschmer et al. 2010). These include the stimulation of neutrophil chemotaxis, activation of neutrophils, and promotion of cytokine release (Wang et al. 2007; Kretschmer et al. 2010). Whether the pro-inflammatory features of PSMs form part of a bacterial pathogenesis program or whether FPR2 evolved to recognize PSMs as pathogen-associated patterns is not yet understood.

Aside from pathogenesis, PSMs appear to have evolved to fulfill different, non-infectious functions as commensal organisms on the skin and mucous membranes of humans and other vertebrates (Periasamy et al. 2012). This notion is supported by their common surfactant-like properties, which likely assist in emulsifying nutrients in the oily environment of skin glands. Furthermore, in vitro research has shown that PSMs contribute to the gliding of staphylococci on surfaces, and thus presumably help in colonization and surface spreading (Tsompanidou et al. 2013). PSMs also may have antimicrobial properties, in particular toward *Streptococcus pyogenes* (Cogen et al. 2010; Joo et al. 2011), although the extent to which these properties contribute to inter-bacterial competition is unknown.

The production of PSMs is under strict control of the staphylococcal quorum-sensing system Agr (Vuong et al. 2004; Queck et al. 2008; Vuong et al. 2003). Agr controls many staphylococcal toxins via an intracellular, regulatory

RNA, called RNAIII (Novick et al. 1993). In contrast to other Agr-regulated targets, the *psm* genes are not regulated by RNAIII, but directly by the response regulator AgrA, which is upstream of RNAIII in the regulatory cascade. This underlines the crucial importance of PSMs for staphylococcal physiology and suggests that the quorum-sensing control of PSM expression preceded that of other quorum-sensing controlled toxins during the evolution of staphylococci as pathogens (Queck et al. 2008).

All PSM peptides are secreted as the primary translation product with an N-terminal N-formyl methionine, which is a characteristic feature of ribosomal bacterial protein synthesis (Cheung et al. 2014). In only a fraction of the secreted peptide is the N-terminal N-formyl methionine removed by the cytoplasmic enzyme N-deformylase. This indicates that secretion of PSMs does not occur via a canonical system, such as Sec-dependent transport, but requires a dedicated secretion system.

Peptide ABC Transporters in Staphylococci

Many secreted staphylococcal peptides are bacteriocins, such as the lanthionine-containing post-translationally modified antimicrobial peptides called lantibiotics (Gotz et al. 2014), and are often exported by dedicated ABC transporters (Otto and Gotz 2001). Examples are the lantibiotic secretion systems of the EpiT prototype (Peschel et al. 1997), with one protein comprising both membrane-spanning and ATPase parts, and the lantibiotic producer immunity systems of the EpiFEG prototype, which consist of two different membrane-spanning proteins and one ATPase, assumed to form a heterodimer with two identical ATPases (Peschel and Gotz 1996; Otto et al. 1998). These occur in the genetic loci of the staphylococcal lantibiotic-producing systems, such as those encoding the biosynthetic loci for epidermin, gallidermin, aureodermin (Bsa), or nukacin. Often, there are dedicated exporters for secretion and producer immunity, with the latter presumably working by constant removal of the membrane-damaging lantibiotic from the membrane; but there also appears to be evidence for a double function of lantibiotic ABC transporters in both immunity and secretion. In those cases, the ABC transporter likely can accept the substrate entering the membrane layer from either the outside or inside of the cell (Hille et al. 2001).

Discovery of the Pmt System

To identify the hypothetical secretion system in charge of secreting PSMs, our laboratory undertook several different approaches, which included transposon mutagenesis. None of the putative candidates identified by transposon mutagenesis showed reduced PSM export after more detailed investigation that comprised the construction of directed gene replacement mutants and measurement of PSM

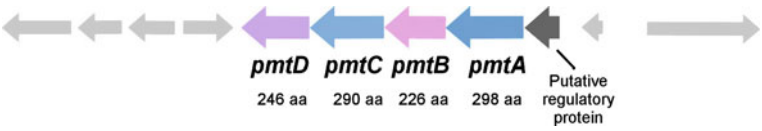

Fig. 2 Genes encoding the Pmt transporter. The *pmt* genes form an operon of four genes. The *pmtA* and *pmtC* genes code for the ABC transporter ATPase parts, the *pmtB* and *pmtD* genes for the membrane-spanning proteins. In front of the *pmt* genes is a putative regulatory protein of the GntR-type. The length of the respective protein products is noted (aa, amino acids)

production in the culture filtrates by high performance liquid chromatography/mass spectrometry (HPLC/MS) (Joo and Otto 2014).

We then hypothesized that expression of the putative PSM export system is under similar regulation as PSM production itself. As the major known regulator of PSM production is Agr, we revisited results that we had achieved previously on Agr-regulated targets in *S. aureus* (Cheung et al. 2011), and filtered the results for systems potentially being involved in export functions. This led to the identification of an ABC transporter system that was Agr-regulated. Hypothesizing that it is involved in PSM export, we named it Pmt for PSM transporter. The Pmt system consists of two genes encoding membrane-spanning proteins and two genes encoding ATPases. In addition, there is a putative regulatory gene in front of the *pmt* genes (Chatterjee et al. 2013) (Fig. 2).

To verify that the Pmt system is in fact involved in PSM export, we attempted to construct gene deletion mutants, in which the entire *pmt* operon is deleted, in strains LAC (USA300) and MW2 (USA400). All these attempts failed, or led to a forced mutation in the *agr* system, abrogating PSM production. These findings suggested that the Pmt system is essential when PSM peptides are produced. Therefore, we attempted to delete the *pmt* genes in strains in which all *psm* genes were previously deleted (Joo et al. 2011), which proved successful. Investigation of those *psm*/*pmt* deletion strains with plasmid-based expression of specific PSMs showed that the Pmt system exports all PSM peptides in *S. aureus*. Furthermore, heterologous expression in *Lactococcus lactis* demonstrated that the Pmt system is sufficient for PSM export (Chatterjee et al. 2013).

Features of the Pmt System

As mentioned above, some PSMs have antibacterial activity, for example toward *S. pyogenes*, but they also exhibit such activity to a certain extent toward staphylococci as PSM producers (Joo et al. 2011). Similar to the lantibiotic export systems, which in addition to their export function may also be involved in producer immunity, likely by accepting their substrate from both the inside and outside

spaces, the Pmt system conferred immunity to PSMs added to the extracellular space (Chatterjee et al. 2013). These included PSMs of the producing organism *S. aureus* but also PSMs of *S. epidermidis*, indicating that the Pmt system confers producer immunity and may function as a protection against PSMs produced by other staphylococci during the co-colonization of human epithelia.

The Pmt system appears to have a high specificity for PSMs, as no other staphylococcal proteins or peptides appeared to be exported by Pmt (Chatterjee et al. 2013). The considerable divergence in PSM primary amino acid sequences suggests that the Pmt exporter recognizes other structural features of PSMs, among which their common secondary structure of pronounced α-helicity and amphipathy is the most likely determinant. This notion is supported by recent unpublished findings in our laboratory indicating that the Pmt system also confers reduced susceptibility to the antimicrobial activity of selected cationic antimicrobial peptides, such as LL-37, which share common structural features (Peschel and Sahl 2006).

Together, the features of the Pmt system, most notably its apparent acceptance of substrate from the intra- and extracellular space, are in accordance with the following hypothetical mechanism also suggested for other ABC transporters that export membrane-active substances (Sharom 2014). The binding site of the Pmt exporter likely is positioned at a location exposed to the membrane bilayer, from which it exports PSMs that have inserted into the membrane, either by what has been called a "vacuum cleaner" or "flippase" mechanism—depending on whether the substrate is expelled to the outside aqueous phase or only flipped to the outer membrane layer. Pmt thus functions by constantly removing PSMs from the intramembrane space and expelling them to the extracellular space, independently of the original (outside or inside) location of PSMs. As illustrated in Fig. 3, current knowledge of the mechanism of ABC transporters and the features of Pmt suggests a model in which the PSM ligand is accepted in a state corresponding to the inward-open state of ABC transporters, however, with the ligand-binding site within the membrane rather than the cytoplasmic space. Transition to a closed state triggers binding of ATP, release of the ligand, and formation of a nucleotide-bound outward-open state (possibly via an additional step including an outward-occluded state). ATP hydrolysis then causes the initial open state to form again, completing the transport cycle, possibly with an outward-occluded intermediate state (Choudhury et al. 2014). Notably, this mechanism is currently hypothetical, yet underscores the need for structural and mechanistic studies.

Finally, an important and exceptional feature of the Pmt system is its essentiality for *S. aureus* growth and survival when PSMs are produced (Chatterjee et al. 2013). In the absence of Pmt, PSMs accumulate in the intracellular space and lead to growth arrest and significant abnormalities in cell shape and division. This explains the problems we encountered when trying to identify the PSM export system using transposon mutagenesis approaches and indicates exceptional value of the Pmt exporter as a target for anti-staphylococcal therapy.

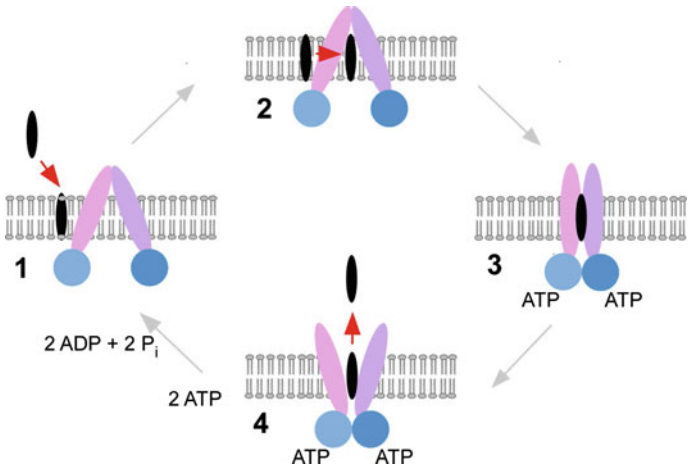

Fig. 3 Putative mechanism of PSM export by the Pmt ABC transporter. The four principal, hypothetical stages in PSM export by the Pmt system are shown, assuming a "vacuum cleaner" mechanism. *1* PSMs integrate into the membrane. Pmt is in an inward-facing open state. *2* A PSM molecule binds to the Pmt ligand-binding site from within the membrane. *3* This triggers ATP binding by the ATPase proteins and a conformational change to a hypothetical outward-occluded state. *4* The PSM molecule is released to the aqueous outside environment and the original, inward-facing state is re-assumed under hydrolysis of ATP

Pmt—A Target for Antimicrobial Therapy?

In a time of ever-increasing antimicrobial resistance, alternative strategies to conventional antibiotics are eagerly sought. These include, in particular, the direct targeting of virulence mechanisms (Alksne and Projan 2000). Judging from the key importance of PSMs for acute staphylococcal infections, the Pmt transporter represents an attractive target to degrade staphylococcal virulence (Chatterjee and Otto 2013). Notably, it offers the potential to suppress PSM production entirely using a single target. In contrast, alternative approaches of interfering with PSM production or function such as by active or passive immunization are inherently problematic due to the multitude and diversity of PSMs.

In addition, the fact that suppressing Pmt function would lead to intracellular accumulation of PSMs and thus a direct impact on cell viability makes Pmt an exceptional target, as anti-Pmt drugs would suppress both virulence and viability. Drugs blocking Pmt may promote mutations in the *agr* system, as has happened during our efforts to delete the *pmt* genes under conditions of PSM production. However, this again may attenuate virulence, as Agr not only strictly regulates PSM production but also that of many other staphylococcal toxins (Cheung et al. 2011). In fact, there have been multiple recent studies re-evaluating Agr as a drug target and proposing specific drugs interfering with Agr (Sully et al. 2014; Khodaverdian et al. 2013; Wright et al. 2005).

Outlook

With the Pmt system only discovered recently, structural and mechanistic data on Pmt are still missing. Deciphering the mechanism of Pmt export and the precise composition and structure of the Pmt membrane protein complex will be of major interest not only from a basic science point of view, as mechanistic details of peptide ABC transporters of the Pmt type are not yet well understood, but also because the structure of Pmt may permit modeling of potential inhibitors. Ideally, such inhibitors of Pmt would interfere with substrate binding or export at the Pmt-specific parts. Interfering with the ATPase domains, which are quite well conserved among different ABC transporters including those of vertebrates, comes with the inherent problem of probable toxicity to humans (Dantzig et al. 2003; Crowley et al. 2010). As a corollary, drug design against Pmt function would thus best proceed with sophisticated screens not only targeted at inhibiting the ATPase functionality of the Pmt complex, but also at substrate binding and export.

Another open question is whether Pmt also confers resistance to antimicrobial peptides, which initial results in our laboratory suggest, and whether this potential additional capacity of Pmt increases bacterial survival in the human host independently of PSM production. In-depth investigation of the substrate specificity of Pmt would also allow more insight into its mechanism.

Finally, Pmt function has so far only been investigated in *S. aureus* strains with considerable PSM production (in the community-associated methicillin-resistant strains LAC and MW2). The impact of Pmt function on bacterial survival in vivo in strains that produce lower amounts of PSMs may be more limited, but may still influence the potential resistance to AMPs. Furthermore, Pmt function remains to be investigated in staphylococcal species other than *S. aureus*, in all of which Pmt is conserved.

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ABC Exporters from a Structural Perspective

Markus A. Seeger, Enrica Bordignon and Michael Hohl

Abstract ABC exporters belong to the ABC transporter superfamily and are found in all organisms. Since ABC exporters are involved in drug efflux and associated with frequent hereditary diseases such as cystic fibrosis, they are of direct medical interest. Despite 40 years of research on ABC exporters, many key questions regarding their molecular mechanism remain unclear or are under debate. The youngest decade of ABC exporter research has produced a dozen full-length ABC exporter structures. While the overall fold is highly conserved, the observed conformations are remarkably diverse. In this review, the crystallographic ABC exporter snapshots are assembled into a photo story and the mechanistic implications derived from these crystal structures are critically discussed.

What Are ABC Exporters?

ABC transporters are divided into ABC importers found exclusively in bacteria and ABC exporters present in all organisms. Whereas ABC importers are further subdivided into three major types (see Chapter contributed by Bert Poolman and Dirk Slotboom), ABC exporters are regarded as one subfamily of ABC transporters (Fig. 1) (ter Beek et al. 2014). The differentiation into importers and exporters has been made based on the directionality of active transport; whereas the ABC importers are mainly responsible for nutrient uptake into bacterial cells, ABC exporters are commonly involved in the extrusion of substances from the cis- to the trans-side of the lipid bilayer, in which the cis-side corresponds to the compartment where ATP is consumed. There are, however, notable exceptions to this general rule: CFTR (ABCC7)

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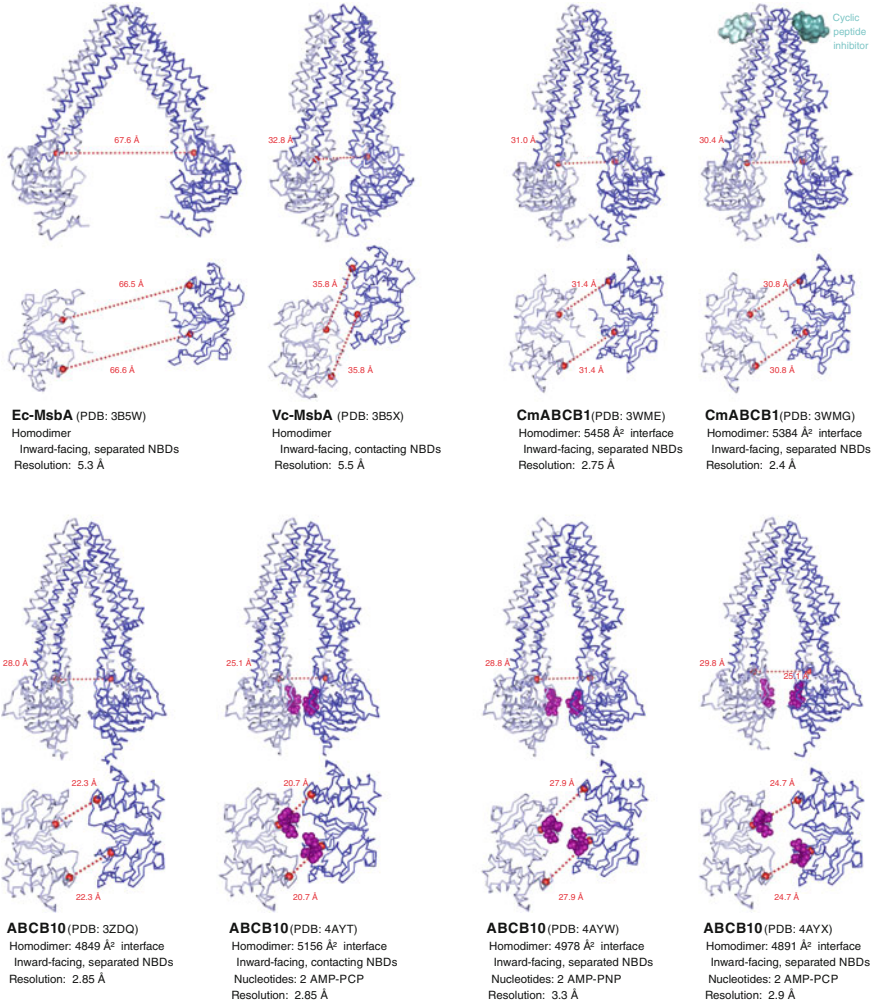

Fig. 1 ABC exporter structures at a glance. In the *top row*, the transporters are viewed along the membrane plane. In the *bottom row*, the NBD pair is viewed from the cell exterior. Inward-facing homodimeric ABC exporters are shown in *dark* and *light blue*. Inward-facing heterodimeric ABC exporters are shown in *blue* and *green* (irrespective of whether they are composed of one or two polypeptide chains). Outward-facing ABC exporters (which are all homodimeric) are shown in *black* and *gray*. Bound nucleotides are depicted as *purple spheres* and bound substrates as *orange spheres*. In the transporters' side view, the distances between the coupling helices—measured between the C_α-atoms (highlighted as *red sphere*) of the residues corresponding to G201 (TM287) and G225 (TM288) of TM287/288—are indicated by *red dotted lines* and *labels*. In the NBDs' top view, the distances between the C_α atoms of the Walker A lysine and the ABC signature motif serine are indicated by *red lines* and *labels*. In case several dimers were present in the asymmetric unit of the corresponding crystal, the dimer containing polypeptide chain A was analyzed. When similar structures were available with different ligands, the *bottom panels* show the corresponding ligands (e.g., for Na-Atm1 and Sc-Atm1). The area of the homo- or heterodimeric interface between the two ABC half-transporters was calculated using

◀the PISA server (http://www.ebi.ac.uk/msd-srv/prot_int/cgi-bin/piserver). Nucleotides and magnesium ions mediating additional dimer contacts were not included in the interface calculations. An NBD–NBD interface larger than 200 Å^2 was interpreted as existing contact. For Vc-MsbA, the NBD–NBD contact area could not be determined (because only the C_α-atom positions are given in the PDB), but due to the close distances between C_α atoms of the two NBDs, the contact area is certainly larger than 200 Å^2

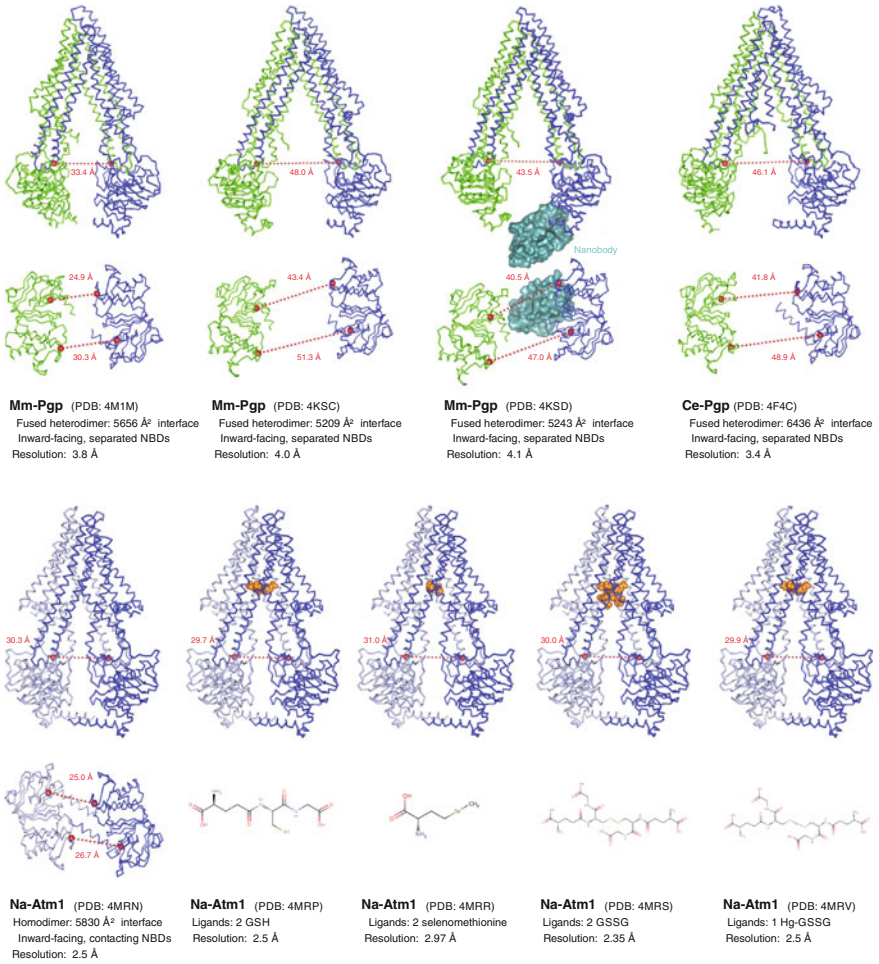

Fig. 1 (continued)

is an ATP-gated chloride channel (Linsdell 2014), SUR1 is a nucleotide-sensing regulator of its associated potassium channel Kir6.2 (Aittoniemi et al. 2009), and ABCA4 in fact transports N-retinylidene-phosphatidylethanolamine from the trans- to the cis-leaflet of disk membranes (Quazi et al. 2012) and therefore strictly speaking is an importer with an ABC exporter fold. When we deal with ABC

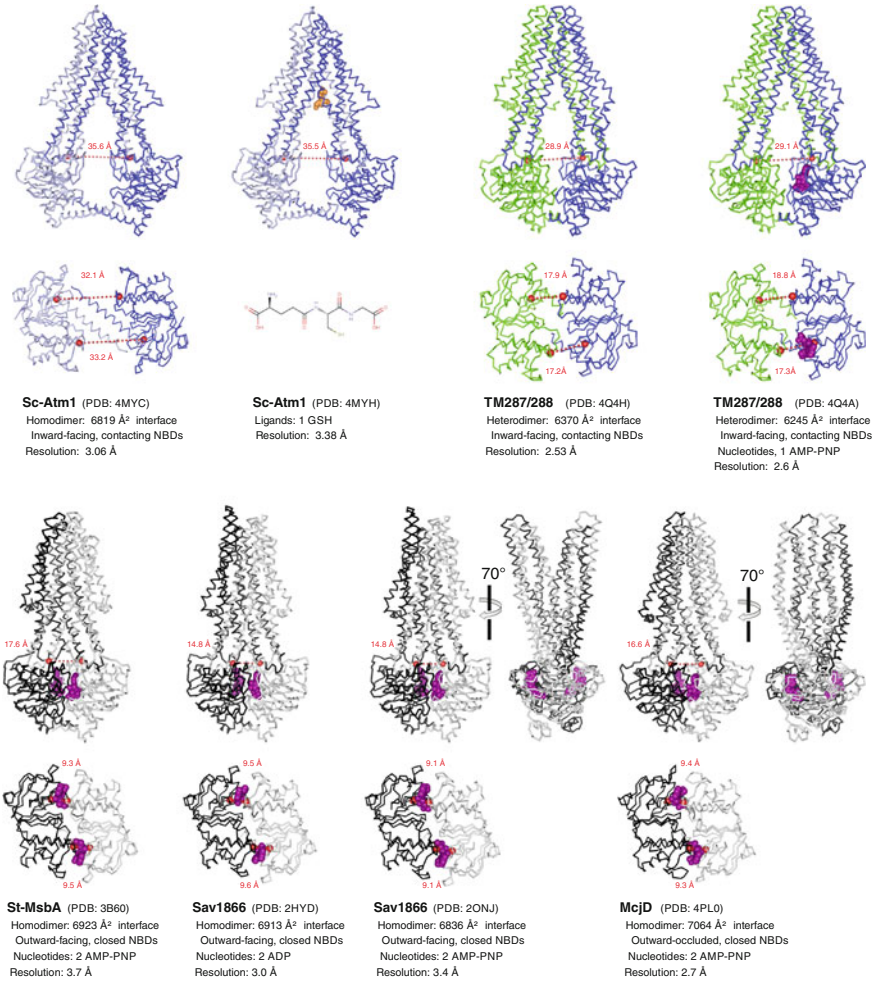

Fig. 1 (continued)

exporters in this chapter, we actually mean the ABC exporter fold and not necessarily the direction of transport. ABC exporters minimally consist of four domains, two transmembrane domains (TMDs) each containing six transmembrane helices [an exception is, for example, the macrolide transporter MacB with only four transmembrane helices per TMD (Kobayashi et al. 2003)] and two nucleotide-binding domains (NBDs). Whereas the TMDs are phylogenetically diverse in order to recognize and translocate the various substrates, the NBDs contain highly conserved sequence motifs including the eponymous ABC signature motif (Jones and George 1999). In addition to these core domains, a large variety of cytoplasmic, extracellular, and transmembrane-spanning domains have been described for ABC exporters, which play various roles in regulation of the core transporter and recognition of other

protein-binding partners. Well-known examples of such additional domains are the R-domain of CFTR (Rich et al. 1991), the TMD0-domain of TAP1/2 (Koch et al. 2004), and the periplasmic domain of MacB needed to recognize the periplasmic membrane fusion protein MacA (Xu et al. 2009). Since structural studies on ABC exporters do not go beyond the four core domains, we shall concentrate on these in this chapter and summarize the current structural knowledge about how two NBDs and two TMDs coordinate uphill pumping of substrates.

The NBD Era

From a historical perspective, structural biology on ABC exporters can be divided into two major eras: the time before and the time after the Sav1866 structure. Between the years 2001 and 2006, studies on ABC exporters focused on NBDs including those of the first NBDs of TAP1/2 (Gaudet and Wiley 2001; Procko et al. 2006), CFTR (Lewis et al. 2004), and MRP1 (Ramaen et al. 2006) and of the hemolysin transporter HlyB (Schmitt et al. 2003; Zaitseva et al. 2005, 2006). These structures—together with NBD structures of ABC importers and ABC-type NBDs involved in functions other than transport—were important to understand the function of the ABC transporters' ATP consuming motor. The collective gain of knowledge derived from these studies was that NBDs dimerize in a head-to-tail fashion upon binding of two ATP molecules and dissociate as a consequence of ATP hydrolysis. This led to the formulation of the ATP switch model (Higgins and Linton 2004), in which the physical motions generated at the NBDs by ATP binding and consumption are coupled to the TMDs to provide alternating access to transport substrates accompanied by a concomitant affinity switch (typically a high affinity site at the cis-side of the membrane and a low affinity site at the trans-side) to permit transport against a concentration gradient. To fulfill their function, NBDs contain a number of highly conserved sequence motifs responsible for nucleotide binding, hydrolysis, and interdomain communication:

(i) The A-loop contains an aromatic residue, which stabilizes the adenine moiety of the bound nucleotide by π-stacking interactions.
(ii) The Walker A motif wraps around the γ-phosphate of ATP and its conserved lysine plays a role in ATP binding and ATP hydrolysis.
(iii) The ABC signature motif plays a key role in the ATP-induced dimerization, in that it sandwiches the bound ATP molecule together with the Walker A motif of the opposite NBD at the dimer interface.
(iv) The Walker B motif contains the catalytic glutamate, which in most but not all ABC transporters is indispensable for ATP hydrolysis.
(v) The catalytic Walker B glutamate is assisted by a conserved histidine of the switch loop.
(vi) The Q-loop glutamine is responsible for the molecular communication with the TMDs.

(vii) The D-loop aspartate plays a key role in the cross-communication between the two ATP-binding sites of the NBD dimer.

Sav1866—A Structure Which Coined the ABC Exporter Field

In the time period of the first NBD structures, two full-length structures of the lipid A transporter MsbA were published, which however—as it became evident with the publication of the Sav1866 structure—were wrong and needed to be retracted. For this reason, the publication of the Sav1866 structure solved at 3 Å resolution did not only mark one of the major steps in understanding ABC exporters at the structural and functional level, but the shock waves caused by the retraction of the MsbA structures reached the entire X-ray crystallography community and in particular the membrane protein field, where the interpretation of X-ray data is often pushed to its limits.

Sav1866 is a homodimeric ABC exporter stemming from *Staphylococcus aureus* and is built up from two half-transporters each comprising a TMD–NBD fusion. Sav1866 transports Hoechst 33342 and ethidium when overexpressed in *Lactococcus lactis* (Velamakanni et al. 2008). The transporter was solved in its outward-oriented state with two nucleotides sandwiched at the NBD–dimer interface (Dawson and Locher 2006). In the initial structure, two molecules of ADP were bound at the NBD interface, which was unexpected because the NBD dimer is anticipated to disengage as a consequence of ATP hydrolysis. However, Sav1866 was also co-crystallized with two bound AMP-PNP molecules in the same crystal form (Dawson and Locher 2007), which was then compatible with the generally accepted ATP switch model of the transport cycle (Higgins and Linton 2004). In Sav1866, the structural organization of the TMDs was revealed for the first time for an ABC exporter. The biggest surprise was that the transmembrane helices 4 and 5 forming the intracellular loop 2 (ICL2) of one half-transporter extensively interact with the opposite NBD via its coupling helix. This unexpected swap had not been anticipated prior to the Sav1866 structure and illustrates the power of X-ray crystallography to facilitate groundbreaking discoveries. Later, the swap was confirmed by cross-linking experiments for a number of ABC exporters (Zolnerciks et al. 2007; Federici et al. 2007) and by the structures of additional 11 ABC exporters (vide infra). The outward-facing cavity is confined by two hexa-helical bundles each consisting of TM1 and TM2 of one protomer and TM3-6 of the other one, which diverge into two separate wings at the trans-side of the lipid bilayer (Dawson and Locher 2006). The cavity is shielded from the inner membrane leaflet, but accessible from the outer leaflet and the extracellular space. Hydrophobic substrates might therefore exit the cavity floating in the trans-side of the lipid bilayer. Due to extensive intertwining of the TMD's helices, the interface between the two half-transporters covers an impressive area of 6913 $Å^2$ in ADP-bound Sav1866 and

more than 4800 $Å^2$ even in case of inward-facing structures with completely separated NBDs (vide infra). Thus, according to the currently known ABC exporter structures the dimer is kept together in every step of the transport cycle via the TMDs. The Sav1886 structure for the first time provided a structural rationale for the coupling between the NBDs and the TMDs; these domains are connected via the coupling helices, which transmit mechanical movements generated at the motor domains by ATP binding and hydrolysis to the TMDs to fuel alternation between the inward- and outward-facing state (Dawson et al. 2007).

The MsbA Trio

In 2007, three (corrected) MsbA structures from *Escherichia coli* (Ec), *Vibrio cholerae* (Vc), and *Salmonella typhimurium* (St) were presented (Ward et al. 2007). MsbA flips lipid A—a precursor of lipopolysaccharides—across the inner membrane and is essential in *E. coli* (Doerrler et al. 2001). Although the three MsbA proteins are closely related at the sequence level (but not identical), the conformations of the three structures differ drastically. The 3.7 Å St-MsbA structure depicts an outward-oriented transporter with bound AMP-PNP akin to Sav1866. The structures of Ec-MsbA (5.3 Å) and Vc-MsbA (5.5 Å) have the shape of an inverted V with the NBDs far apart from each other (Ec-MsbA) or twisted against each other (Vc-MsbA) and the TMDs enclosing a wide-open inward-oriented cavity. The inward-facing structures reinforced the structural role of the domain-swapped ICL2 to represent the handle on which the NBDs convey mechanical force onto the TMDs. The structures were found to be in good agreement with electron paramagnetic resonance (EPR) studies of the conformational cycling of Ec-MsbA, suggesting that such large conformational changes are not only observed in crystals, but also in detergent solution and upon protein reconstitution into proteoliposomes (Borbat et al. 2007; Zou et al. 2009; Mittal et al. 2012). Whereas crystallographic snapshots depict a single "frozen" conformation of a protein, pulsed EPR techniques such as DEER [double electron–electron resonance, also known as PELDOR (Jeschke 2012)] capture the proteins' conformational ensemble in detergents or lipids trapped at cryogenic temperatures. DEER showed considerable molecular disorder (related to fluctuations at physiological temperature) between the NBDs of inward-facing apo Ec-MsbA and a comparatively rigid outward-facing state with closed NBDs. DEER studies on the *L. lactis* ABC exporter LmrA confirmed large NBD fluctuations; however, in contrast to Ec-MsbA, broad distance distributions were also reported for the TMDs of apo LmrA, indicating substantially more flexibility in the TMDs of LmrA (Hellmich et al. 2012). The DEER data were further supported by a hydrogen/deuterium exchange (HDX) study coupled to mass spectrometry on the homodimeric ABC exporter BmrA of *Bacillus subtilis* (Mehmood et al. 2012). The analysis showed marked differences of amide backbone HDX between apo BmrA adopting the inward-facing state and a BmrA E504A mutant which completely shifts to the

outward-facing state in the presence of MgATP. Not only the NBD regions directly involved in nucleotide binding were more prone to HDX in the apo state, but also the entire NBDs and the intracellular loops were affected, lending support to the complete separation of the NBDs of BmrA in detergent solution. Since solvent accessibility calculated based on homology models could not account for the experimentally observed HDX differences for the apo state, it was concluded that the intracellular regions of BmrA are highly dynamic while the transporter adopts its inward-facing state. Interestingly, the coupling helices and the grooves of the NBDs, which accommodate the coupling helices, were not protected from HDX in the apo state, suggesting their transient decoupling from the NBDs (Mehmood et al. 2012).

Two Inward-Facing P-Glycoprotein Structures

The mouse P-glycoprotein (*Mus musculus* Pgp, Mm-Pgp) structure solved at moderate resolution of 3.8 Å marked the first ABC transporter structure of eukaryotic origin and the first of a full transporter comprising all four domains in one polypeptide chain (Aller et al. 2009). Although the structure needed major corrections later (Li et al. 2014) [and was silently corrected in the Mm-Pgp structure with a nanobody bound (Ward et al. 2013)], apo Mm-Pgp clearly exhibits an inward-facing state with completely separated NBDs. Two cyclic peptides, which both inhibit transport of other compounds, were shown to bind close to the trans-side of the large inward-oriented cavity (Aller et al. 2009). These inhibitor-bound structures were solved at 4.4 Å resolution (i.e., a resolution at which unambiguous placing of side chains is in principle impossible). From the corrected apo Mm-Pgp structure it is evident that the region of Mm-Pgp harboring the side chains postulated to interact with the cyclic peptides contains many out-of-register errors (Li et al. 2014). This casts severe doubts on the correctness of the molecular details showing how the cyclic peptides interact with Mm-Pgp (Aller et al. 2009). However, based on the anomalous signals obtained for three selenium atoms contained in the cyclic peptide inhibitors, it is at least fairly certain that the compounds indeed bind to the transporter at the postulated region (Aller et al. 2009). In 2012, the apo structure of the *Caenorhabditis elegans* P-glycoprotein (Ce-Pgp) was solved at 3.4 Å resolution in its inward-facing state with completely separated NBDs, which are even farther apart than in the initial Mm-Pgp structure (Jin et al. 2012). The Ce-Pgp structure was in part incompatible with the closely related Mm-Pgp structure, in particular at the coupling helix of ICL2 [the regions in question have been corrected in the revised Mm-Pgp structure (Li et al. 2014)]. The TMDs of Ce-Pgp featured two structural novelties. First, one lateral entry site of the cavity appears to be blocked by two short helices preceding the ordinary six transmembrane helices of TMD1. The functional role of this N-terminal extension remains unclear and these helices likely need to be squeezed out of the cavity during the transition to the outward-oriented state. Second, the other lateral entry to the cavity contains discontinuous transmembrane helices, which might play a role

in providing access for hydrophobic Ce-Pgp substrates embedded in the inner leaflet of the membrane to the cavity. The Ce-Pgp structure made it finally clear that inverted V-shaped structures for nucleotide-free ABC exporters are rather the rule than the exception. Two further Mm-Pgp structures, one wide open and one with an inhibiting nanobody bound to NBD1, confirmed this notion (Ward et al. 2013). A combined DEER and molecular dynamics (MD) study on Mm-Pgp suggested considerable dynamics of the inter-NBD distance (Wen et al. 2013). The DEER traces of Mm-Pgp labeled at the NBDs indicated broad distance distributions representing large ensembles of conformational states with distances being longer or shorter than the expected distance based on the initial Mm-Pgp structure. The MD simulations suggested that the motions originate from kinks in the TMD region at glycine and proline containing positions [but not from detachment of the coupling helices from the NBDs as suggested in Mehmood et al. (2012)], which are amplified along the long intracellular loops and give rise to large fluctuations seen for the NBDs (Wen et al. 2013). Unfortunately, this MD study was performed using the initial, erroneous Mm-Pgp structure.

Two Well-Resolved Inward-Facing ABC Exporters of Eukaryotic Origin

The first ABC transporter structure of human origin was that of ABCB10, a homodimeric mitochondrial ABC exporter solved in four structurally similar but clearly not identical inward-facing states, three of them at resolutions better than 3 Å (Shintre et al. 2013). ABCB10 plays a role in hemoglobin maturation during erythropoiesis and in protection against oxidative stress. However, the transport substrate is currently unknown. In contrast to the structures of P-glycoprotein and apo MsbA, the NBDs are less separated and even touch each other in one case (PDB: 4AYT). ABCB10 was solved in its apo state as well as in the presence of AMP-PNP or AMP-PCP. Against the expectation, the presence of nucleotides did not result in NBD closure. Because DEER studies on ABCB10 are lacking, it remains to be shown whether the detergent-purified protein can at all adopt its outward-facing state in the presence of the two nucleotide analogs used for crystallization. Several detergent and cardiolipin molecules were found to bind to the transmembrane helices. In two of the crystal forms (PDB: 4AYT and PDB: 4AYX), a lateral opening at the TMDs provides access from the middle of the lipid bilayer to the inward-facing cavity. An alkyl chain of a cardiolipin molecule traverses through this opening, indicating that it is sufficiently large to permit passage for small hydrophobic molecules. The functional relevance of this portal however remains unclear, mainly because it is oriented toward the trans-side of the lipid bilayer, which for exporters would be an exit portal. A sequence alignment of different ABCB10 homologs revealed a highly conserved patch of residues located at the lower half of the inward-facing cavity between TM2 and TM3, which are

likely playing a key role in the recognition of the still unknown substrate. Interestingly, the location of this patch partially overlaps with the glutathione (GSH)-binding site of the iron transporter Atm-1 (vide infra).

With the structure of the homodimeric P-glycoprotein homolog called CmABCB1 from the eukaryotic organism *Cyanidioschyzon merolae*, another nucleotide-free inward-facing ABC exporter structure was reported exhibiting intermediate NBD separation (Kodan et al. 2014). This is clearly the ABC exporter structure with the highest resolution to date (2.4 Å according to the authors, but owing to a high I/σI value of 4.8 in the last resolution shell, they could have extended their data even further). An in vitro selected macrocyclic peptide—called aCAP—was used as crystallization chaperone (Hipolito and Suga 2012). Interestingly, aCAP was not itself involved in crystal contacts (strictly speaking it did not act as a true chaperone), but rather ties TM2 and TM6 together at the extracellular side. Based on the structures of Sav1866 and St-MsbA, TM2 and TM6 are anticipated to separate when the two wings diverge during the transition to the outward-facing state; aCAP therefore appears to stabilize the inward-facing conformation of CmABCB1. ATPase activities of CmABCB1 were strongly inhibited in the presence of aCAP, indicating that productive NBD dimers are less likely to form when the opening of the wings is blocked by a molecular clamp. Expressed in yeast, CmABCB1 transports multiple drugs typically recognized by human or mouse P-glycoprotein. Extensive mutagenesis experiments suggested the presence of a multidrug-binding site consisting of aromatic and aliphatic residues at the narrow ceiling of the cavity. One tyrosine residue (Y358) was of particular importance for Rhodamine 6G transport. Unfortunately, it appeared impossible to co-crystallize CmABCB1 with its substrates (Kodan et al. 2014). In contrast to wide-open inward-facing structures, the cleft between TM4 and TM6 of CmABCB10 is too narrow to permit substrate access from the inner membrane leaflet to the cavity. However, in analogy to TM10 of Ce-Pgp, TM4 of CmABCB1 is highly flexible, as judged based on high B-factors in this region in the crystal structure. Due to TM4 flexibility, entry of membrane-partitioned hydrophobic substrates appears feasible via this intramembranous gate. TM4 became more rigid upon mutation of one glycine and two alanines to valines, as was demonstrated by reduced B-factors in this region in the structure of the corresponding triple mutant. The triple mutant was no longer capable of transporting substrates, which lent support for the functional importance of flexibility of TM4 (Kodan et al. 2014).

Unambiguous Visualization of Substrate Binding to Metal ABC Exporters

Crystal structures of two ABCB7 homologs—one from the Gram-negative bacterium *Novosphingobium aromaticivorans* (called Na-Atm1) (Lee et al. 2014) and one from *Saccharomyces cerevisiae* (called here Sc-Atm1) (Srinivasan et al. 2014)

—were solved at resolutions of 2.4 and 3.06 Å, respectively. The mitochondrial ABC exporter ABCB7 is essential for the biogenesis of cytosolic and nuclear ironsulfur (Fe/S) proteins and is presumably responsible for the transport of Fe/S clusters out of mitochondria, the cellular compartment where they are assembled. Na-Atm1 and Sc-Atm1 are homodimeric ABC exporters and their inward-facing structures were solved in the absence of nucleotides. Although the Walker A motif and the ABC signature motif are far apart from each other, the NBDs are kept together via their C-terminal helices. The C-terminal helix is particularly long in Sc-Atm1, in which the C-terminus almost reaches over to the Walker A motif of the opposite NBD. Truncation of the entire C-terminal helix of Sc-Atm1 resulted in a somewhat reduced activity of the transporter (as measured for ^{55}Fe incorporation into the cytoplasmic Fe/S-protein Rli1), but reduction of transport might also be owing to decreased expression of truncated Sc-Atm1. Although the physiological substrate of these ABC exporters remains speculative, both Na-Atm1 and Sc-Atm1 were co-crystallized with glutathione (GSH) and its derivatives and it was suggested that Fe/S clusters are transported in a GSH-liganded form through Atm1. In Sc-Atm1, no ligand was added for crystallization, but an extra density located in the inward-facing cavity at the level of the inner leaflet of the membrane—which was postulated based on mass spectrometry to correspond to GSH—was apparent in some of the crystals. In the reported Sc-Atm1/GSH co-crystal structure solved at 3.38 Å resolution, GSH was bound to only one of the three half-transporters (TMD-NBD) of the asymmetric unit, indicating subtle asymmetries between the three chains. Residues at a kink of TM6, which are conserved in different ABCB7 homologs, were found to be of particular importance for GSH recognition. Na-Atm1 was co-crystallized with GSH and a veritable series of oxidized GSH derivatives, which all stimulated ATPase activity of the transporter. In contrast to Sc-Atm1, Na-Atm1 binds two molecules of GSH, recognized in a symmetric fashion by both chains of the homodimeric transporter (the asymmetric unit contains the homodimer). The binding location is similar to that of Sc-Atm1 and involves residues of TM3 and TM6 close to helical irregularities. For GSSG (oxidized GSH), two binding sites were observed: a major binding site where also the reduced GSH molecules are bound and a second binding site located further down at the cis-side of the transporter. Compared to the Mm-Pgp inhibitors bound at the ceiling of the cavity, the major GSSG-binding side is around 15 Å closer to the cytoplasmic side of the membrane. Additional co-crystal structures of Na-Atm1 revealed that the major binding site can also accommodate the free amino acid selenomethionine and the mercury–GSH complex S–Hg $(GSH)_2$. The anomalous signals of selenium and mercury of these two binding substrates together with the high-resolution datasets (between 2.5 and 3 Å) achieved for the substrate-bound co-crystals allowed for an unambiguous placing of the bound ligands. The molecular details of transport substrate recognition by Na-Atm1 can therefore be trusted with high confidence. Mutations of residues in the major GSSG-binding site resulted in marked changes of the basal ATPase activities of Na-Atm1 and—even more interestingly—in a loss of substrate stimulation of ATP hydrolysis, reinforcing their functional role in recognizing the substrate and in cross-communication to the NBDs.

Heterodimeric ABC Exporters with a Degenerate ATP-Binding Site

In many heterodimeric ABC exporters one ATP-binding site deviates from the consensus sequence in the catalytically important Walker B glutamate, the switch loop histidine, and the ABC signature motif of the opposite NBD. Thereby one of the two composite ATP-binding sites is catalytically impaired and for this reason called the degenerate site (Procko et al. 2009). Approximately half of the 46 human ABC exporters contain a degenerate site (but many of them not carrying the full set of degeneracies at Walker B, switch loop, and ABC signature), in particular all members of the ABCC family including the clinically important proteins CFTR and SUR1 (Aittoniemi et al. 2009; Gadsby et al. 2006). The first and still only representative of this class of heterodimeric ABC exporters solved at atomic resolution is TM287/288 from the hyperthermophilic bacterium *Thermotoga maritima* (Hohl et al. 2012). In analogy to its close homolog LmrCD of *L. lactis* (Lubelski et al. 2006), TM287/288 is capable of transporting the anticancer drug daunomycin. The TM287/288 structure was first obtained in the presence of AMP-PNP, which was exclusively bound to the degenerate site while the transporter adopted an inward-facing orientation (Hohl et al. 2012). Later, the structure of apo TM287/288 was solved in the same crystal form depicting a very similar state, with conformational changes almost exclusively taking place in the NBDs (Hohl et al. 2014). By contrast to all other inward-facing ABC exporter structures solved thus far, the NBDs of TM287/288 remain connected mainly via the D-loop of the TM288 chain, which interacts with residues of the Walker A motif of the opposite NBDs via multiple hydrogen bonds. The hydrogen-bonding network is larger when AMP-PNP is present at the degenerate site, but remains strong enough to keep the NBDs together also in the apo state (Hohl et al. 2014). The D-loop of the TM287 chain was found to be highly flexible and establishes two hydrogen bonds with the opposite NBD only if the degenerate site is occupied with a nucleotide. Mutating the TM287 D-loop aspartate to an alanine or restricting the D-loop flexibility by a disulfide bond resulted in decreased ATPase activities and diminished drug transport of the TM287/288 homolog LmrCD. A study on TAP1/2, a heterodimeric ABC exporter of the ABCB family responsible for the translocation of peptides from the ER lumen to be loaded on the MHC complex, revealed that if the TAP1 D-loop aspartate is substituted by alanine, the transporter is no longer capable of active uphill transport, but is turned into a facilitator requiring the presence of ADP, ATP, or AMP-PMP for peptide translocation down its concentration gradient (Grossmann et al. 2014). The D-loops therefore appear to be of particular importance for cross-communication between the asymmetric ATP-binding sites of heterodimeric ABC exporters and in TAP1/2 they seem to be also involved in the coupling between the TMDs and NBDs.

TM287/288 could not be co-crystallized with a substrate and therefore, the molecular details of drug recognition remain a hidden secret. However, three xenon atoms were found to bind to hydrophobic patches of the cavity (as shown by the

anomalous signal of Xe), indicating possible regions for interactions with hydrophobic substrates (Hohl et al. 2012). The cavity of TM287/288 is inaccessible for substrates partitioned in the inner membrane leaflet. In contrast to Ce-Pgp (Jin et al. 2012) and CmABCB1 (Kodan et al. 2014), TM4 (of both TM287 and TM288) does not appear to serve as flexible entry portal. Of note, TM287/288 is the only ABC exporter stemming from a hyperthermophile whose X-ray structure has been solved. At elevated temperatures TM287/288 might nevertheless be flexible enough to warrant substrate access from the inner leaflet of the lipid bilayer. Another unique structural feature is found at the elbow helix of TM288, which crosses over to TM4 of ICL4 and thereby might help to further restrict the opening of the cavity and of the NBDs (Hohl et al. 2012).

Very recently, a single-particle electron cryomicroscopy structure of the heterodimeric multidrug ABC exporter TmrAB of the thermophilic bacterium *Thermus thermophilus* was solved at 8.2 Å resolution in the absence of nucleotides (Kim et al. 2014). It represents the first sub-nanometer structure of a transporter solved by this technique and required the selection of TmrAB specific antibody fragments to achieve this resolution. While the electron density did not allow for the placing of side chains, it was of sufficient quality to trace the transmembrane helices and the NBDs. Using the crystal structure of AMP-PNP bound TM287/288 as template, a pseudo-atomic model of TmrAB was constructed. Although the NBDs of TmrAB are slightly more separated compared to TM287/288 and appear to be somewhat twisted along the dimerization plane, they interact with each other such that full NBD separation does not occur. As a consequence of the more pronounced NBD separation, the lateral entry portals to the inward-facing cavity are opened wider in TmrAB than in TM287/288, permitting access for hydrophobic drugs from the inner leaflet of the membrane. NBD contacts in TmrAB reinforce the notion that major differences in terms of the apo conformation between heterodimeric and homodimeric ABC exporters exist. However, the NBD interface may be more stable in TM287/288 and TmrAB than in other ABC exporters due to the fact that these proteins stem from thermophilic organisms. The method of single-particle electron cryomicroscopy has the potential to reveal further relevant states of TmrAB as well as of other ABC exporters without the need to crystallize them. However, the current resolution is still far off from providing molecular details of the polypeptide side chains and it is still a long way to go until substrate binding can be visualized at the atomic scale with this method.

The NBD contacts seen in the TM287/288 structures are seemingly at odds with the ATP switch model in the sense that the NBDs do not fully separate and it is tempting to see them in support of the constant contact model (Jones and George 2009; Senior et al. 1995; George and Jones 2012). The main difference between the ATP switch model and the constant contact model is, that in the ATP switch model both ATP-binding sites “open” at the resetting point of the transport cycle (i.e., a separation between the Walker A motif and the ABC signature motif occurs at both ATP-binding sites such that no nucleotide is sandwiched), whereas in the constant

contact model, one ATP-binding site remains closed at all times (i.e., one nucleotide remains sandwiched between Walker A and the ABC signature motif). As can be seen clearly, the AMP-PNP bound at the degenerate site of TM287/288 is not sandwiched, because it is not in contact with the ABC signature motif of TM288. This also manifests in a C_α–C_α distance between Walker A lysine and ABC signature serine of 17.3 Å in TM287/288, which is considerably longer than in closed NBDs having the corresponding distances in the 9.1–9.6 Å range. Hence, the structure of AMP-PNP bound TM287/288 does not support the constant contact model, but rather is in accord with the ATP switch model. Of note, the ATP switch model does not per se exclude NBD contacts to be sustained upon opening of the ATP-binding sites, provided that at one point of the transport cycle any nucleotide is sandwiched between the Walker A motif and the ABC signature motif. While the constant contact model has not been disproved by crystallography all current structures fit into the ATP switch model, albeit with variable extent of NBD separation at the resetting point of the cycle. Importantly, partial disengagement of the NBDs as seen in the TM287/288 structures permits the coupling helices to move apart from each other by a distance which is sufficiently large for the TMDs to adopt the inward-facing state (Hohl et al. 2012). In other words, large NBD separations as seen in numerous ABC exporter structures are not required to adopt an inward-facing state. Whether the TMDs can still adopt an inward-facing state while one ATP-binding site sandwiches a nucleotide (as it would be the case in the constant contact model) is currently not known, because a corresponding structure directly supporting this model is lacking.

DEER measurements on TM287/288 support the notion that the apo and the AMP-PNP bound state of this ABC exporter are structurally highly similar (Hohl et al. 2014). Nevertheless, at the level of the NBDs, considerable dynamics was observed as a consequence of AMP-PNP binding. It is possible that the two crystal structures of TM287/288 provide a rather static picture because they were crystallized in exactly the same crystal lattice. Importantly, it is evident from DEER that AMP-PNP cannot induce the outward-facing state of TM287/288, which is in agreement with the inward-facing AMP-PNP bound TM287/288 structure, but stands in contrast to DEER studies on MsbA (Borbat et al. 2007; Mittal et al. 2012). DEER studies on BmrCD, a close homolog of TM287/288 from *B. subtilis*, confirmed the notion that AMP-PNP binding is not sufficient to shift BmrCD to its outward-facing state (Mishra et al. 2014). Rather, trapping of the posthydrolytic state in the presence of ATP and vanadate was required to induce this major transition at the TMDs. Based on this observation, it was concluded that the power stroke for transport in heterodimeric ABC exporters is provided by ATP hydrolysis rather than ATP binding, which challenges the ATP switch model at least for the subclass of heterodimeric ABC exporters (Mishra et al. 2014). Whether this is valid for other heterodimeric exporters remains to be elucidated in future work. The DEER studies on BmrCD further revealed that in contrast to the TMDs, which adopt only two major conformations in agreement with the inward-facing structure of TM287/288 and outward-facing structure of Sav1866, the NBDs adopt three major conformational states. In the presence of vanadate trapped ATP, they show a closed

configuration (as in Sav1866), in the presence of AMP-PNP, the NBDs are semi-closed (as in AMP-PNP bound TM287/288), and in the absence of nucleotides the NBDs completely separate akin to the inward-facing conformations of MsbA and P-gp. This stands in contrast to the apo structure of TM287/288 and DEER measurements of nucleotide-free TM287/288 with spin-label pairs introduced at the NBDs comparable to the ones introduced into BmrCD (Hohl et al. 2014). Whether the degree of NBD separation in ABC exporters stemming from thermophilic organisms is modulated by temperature under physiological conditions is a tantalizing hypothesis to be addressed in future studies. Currently, major differences regarding the transport cycle not only appear to exist between homodimeric and heterodimeric ABC exporters, but even between heterodimeric ABC exporters from different bacteria.

A Structure Representing the Outward-Occluded State

The structure of the homodimeric lasso peptide transporter McjD of *E. coli* was solved at a resolution of 2.7 Å in the presence of AMP-PNP and magnesium (Choudhury et al. 2014). McjD adopts a unique conformational state with closed NBDs (as in Sav1866) and an occluded cavity at the TMDs. The state results from a closure at the inner TMD gate mediated by the coupling helices connected to the closed NBDs and a closed (or not yet opened) extracellular gate. In other words, the structure is similar to those of Sav1866 and St-MsbA with the exception that it is not "winged". The dimer interface of McjD measures 7064 $Å^2$, which is larger than that of any other ABC exporter structure. The occluded cavity has a volume of 5900 $Å^3$, which is just about the size of its cognate substrate, the lasso peptide Mcc25. Functional analyses revealed that McjD protects *E. coli* from the toxic effects of Mcc25 production and that a Bodipy-labeled Mcc25 accumulates much slower in cells expressing active McjD as compared to cells expressing the inactive Walker B glutamate to glutamine mutant. Further, it was demonstrated that Mcc25 stimulates the ATPase activity of detergent-purified and reconstituted McjD. Lasso peptide binding to purified McjD was studied by microscale thermophoresis, revealing a substrate-binding affinity of around 100 μM. This number has to be taken with care, because the solubility limit of Mcc25 prevented measurements at saturating concentrations. Regarding the functional relevance of the outward-occluded state, there are still many open questions. First, it remains to be shown whether this state is a specialty of McjD and other microcin transporters or an intermediate transiently adopted by most ABC exporters. Second, DEER or FRET studies on McjD are urgently needed to monitor the conformational dynamics at the extracellular gate; it is well possible that this gate is more flexible than the crystal structure is suggesting. In addition, DEER could provide information about the NBD opening in the absence of nucleotides and might be useful to validate the (low) binding affinity of Mcc25 with an alternative method. While these questions hopefully will be clarified by future studies, the MjcD structure represents

an interesting intermediate, which likely is adopted by many other ABC exporters when switching between the two "extreme" inward- and outward-facing states.

ABC-Challenges for the Future

Which groundbreaking insights can structural biology and structure-based biochemistry and biophysics deliver in the future? Here, we would like to provide a (by no means complete) list of relevant open questions.

(i) Crystallographic description of the transport cycle of ABC exporters.
Crystallographic studies on the maltose transporter (type 1 importer) (Oldham and Chen 2011) and vitamin B12 transporter (type 2 importer) (Korkhov et al. 2014) described multiple conformational states of one and the same transporter as it runs through the transport cycle. Such an in-depth analysis is lacking for ABC exporters, and all currently proposed transport mechanisms are partially based on homology models. The transport cycle minimally includes an inward- and an outward-facing state, with the possible existence (at least based on the McjD structure) of an outward-occluded state. Detailed crystallographic studies are needed to describe one and the same system in its multitude of relevant states. Such studies hold the promise to better categorize different ABC exporter subfamilies, as currently discussed with respect to homo- and heterodimeric ABC exporters (Mishra et al. 2014).

(ii) Wide-open apo structures—are they functionally relevant or detergent artifacts?
The functional relevance of the "teepee"-shaped inward-facing structures is still under debate, because many researchers consider them as crystallization or detergent artifacts, despite the fact that large NBD separation has been observed by DEER in proteoliposomes and nanodiscs in MsbA and BrmCD, respectively (Zou et al. 2009; Mittal et al. 2012; Mishra et al. 2014). Two questions are relevant with regard to this important question. First, "apo" does not really exist in the cell, because millimolar ATP concentrations in the cytoplasm by far exceed the apparent ATP affinity of a typical ABC exporter. This means that a nucleotide-free transporter only exists for short time periods, for example when nucleotides are exchanged at both ATP-binding sites at the same time. The physiologically relevant question is: Which conformational ensemble do ABC exporters adopt while the machine is operating in the native membrane? Recent FRET and DEER studies on Mm-Pgp and BmrCD attempted to experimentally address this question (Mishra et al. 2014; Verhalen et al. 2012). Second, it would be fantastic if one could study the conformational cycle of ABC exporters in the living cell or at least in cell-derived membrane vesicles. Detergent purification may destabilize the transporters and be the reason for NBD separation, even if the protein is subsequently brought back into proteoliposomes or nanodiscs after

purification. According to the current structure gallery, the great majority of inward-facing ABC exporters exhibit separated NBDs. Exceptions are Na-Atm1 and Sc-Atm1, whose NBDs are connected via C-terminal helices and in particular the heterodimeric ABC exporters TM287/288 and TmrAB, whose NBDs remain in rather close contact while the TMDs adopt the inward-facing state. Future studies will hopefully clarify whether the observed differences regarding NBD separation are due to non-physiological artifacts or are indeed a hallmark of different ABC exporter subfamilies.

(iii) Substrate-stimulated ATPase activities—cross-talk between the NBDs and the TMDs
Although many functional and biochemical studies have identified specific residues to play a role in substrate-induced hydrolytic activity, it is still not clarified from a structural perspective how ATP hydrolysis is stimulated in the presence of a transport substrate. Although the Na-Atm1 study provided important structural insights into residues involved in substrate binding, the substrate-free Na-Atm1 structure did not reveal conspicuous structural differences which could explain substrate-induced ATPase stimulation at the molecular level (Lee et al. 2014).

(iv) Orchestration of the transport cycle
The coupling of substrate binding and release with the ATPase cycle is poorly understood. For example, it is not clear for most ABC exporters whether substrate or ATP binds first while the transporter is in the inward-facing state. In addition, studies addressing the transition to the outward-facing state in response to substrates and/or ATP are scarce. Finally, it is not known whether ATP hydrolysis is required for substrate release or simply for the resetting of the transporter back to the inward-facing state. These questions may be tackled in the near future by spectroscopic techniques such as DEER and FRET.

(v) "How many ATP molecules are consumed per transported molecule?"
This simple, recurrent question can still not be answered. The question may be mastered by biochemical transport experiments using polar substrates which do not cross the lipid bilayer spontaneously. More transport studies as the ones recently conducted on TAP1/2 and TAPL (Grossmann et al. 2014; Zollmann et al. 2015) are urgently needed to address this fundamental question.

(vi) The structural and functional role of extra domains
As mentioned in the Introduction, many ABC exporters feature extra domains. These may be integral part of a transporter complex and are likely to modulate the function of the core transport unit by protein–protein interactions or by recruiting yet additional binding partners. Given the large complexity and variability of extra domains in both bacteria and eukaryotes, many novel insights into the biology of larger ABC exporter assemblies are awaiting their discovery.

(vii) The big five
Since a number of ABC exporters are directly linked to hereditary human diseases or play a role in drug efflux from tumor cells and in the distribution of administered drugs in the body, there are (at least) five human ABC exporters

of central interest awaiting to be solved by X-ray crystallography (or any other method, which in future can provide an atomic resolution), which are listed in the following. CFTR, because of its failure due to mutations, leads to one of the most common hereditary diseases, cystic fibrosis, and because it is in fact not a transporter, but a chloride channel. TAP1/2, because of its central role in immunology and because of its well-studied mechanism of transport. Human P-glycoprotein (ABCB1), because it is the most famous multidrug efflux pump. ABCG2, because besides P-glycoprotein it is an important drug efflux pump. Finally SUR1, as it plays an important role in inherited diabetes and has an interesting mechanism in modulating an associated potassium channel. The next book celebrating 50 years of ABC transporter will hopefully contain at least one of these structures and it would be interesting to know already now, whether these structures will be the fruit of "classical" X-ray crystallography or of revolutionary methodologies in structural biology.

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Single Molecule or Ensemble Fluorescence Microscopy Investigations of ABC Transporter Oligomerisation and Dynamics

Kelvin Wong, Nicholas D. Holliday and Ian D. Kerr

Abstract The study of any protein requires investigation not just of that protein in isolation, but the identification and significance of any interacting proteins, as they may be essential for localisation, activity and regulation. For the ATP-binding cassette (ABC) transporters it is no different. Much as we probe into their molecular mechanisms and intramolecular interactions we also need to ensure we unravel their intermolecular interactions. In this chapter we examine how fluorescence microscopy techniques enable us to examine the oligomerisation and dynamics of ABC transporters and offer some perspectives about how such techniques will be used in the future to better understand ABC transporter biology.

Keywords ABC transporter · Fluorescence microscopy · FRET · FRAP · TIRF · Single molecule · BiFC

Introduction

ATP-binding cassette (ABC) transporters are highly dynamic structures with multiple conformational states present during their catalytic and transport cycles (Oldham and Chen 2011). We also know that they are involved in complex, multipartite interactions with other proteins, both membrane-spanning and extra-membranous. The importance of these dynamic changes and interaction networks is essential to understand ABC transporter biology. Fluorescence microscopy, the primary focus of this chapter, is but one weapon in the armoury of biologists studying protein interaction and dynamics, and it would be remiss of us to exclude mention of some of the other techniques that have made significant contributions to these areas of research. In terms of identifying protein–protein interactions, it is well established that co-expression of some ABC transporters with

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interacting partners is required either for trafficking to the cell membrane, or for effective function, or indeed both, and that these assembled complexes can be purified intact by immunoaffinity techniques. Multicomponent bacterial ABC transporters are obvious examples of this (ter Beek et al. 2014; Mourez et al. 1997), but many examples in eukaryotic ABC transporter biology also exist; for example, the trafficking and assembly of a functional sitosterol transporter requires co-expression of both ABCG5 and G8 (Graf et al. 2003). Similarly, the ATP-sensitive potassium channels (K_{ATP}) and transporter responsible for antigen presentation (TAP), both of which are described in more detail subsequently, are regulated ABC protein complexes. As well as these analyses of individual protein–protein interactions there are also genetic screens that have identified putative interaction partners for ABC transporters, and indeed some of these candidates have been followed up by one of the methods we will describe here (Snider et al. 2013). In terms of dynamics, a panoply of in vitro and in silico techniques have been applied to ABC transporters, but space limitations prevent us from anything other than an acknowledgement of their existence in the literature, including elsewhere in this volume. Hereafter, we limit ourselves to the use of fluorescence microscopy techniques to explore ABC transporter dynamics and interactions, acknowledging that the data imparted by these methods augments other biochemical and computational studies. Additionally, we include details of ABC transporter structure and dynamics where knowledge has been imparted by examination of double-fluorophore labelled protein. We have also had to exclude papers describing the use of fluorescent substrates to examine ABC transporter kinetic parameters, but acknowledge that such experiments are now possible at close to single molecule resolution, which will allow determination of turnover number and potentially, stoichiometry (Zollmann et al. 2015).

Fluorescent Tagging

A major consideration at the outset of such work is the nature and location of the fluorescent tag; i.e. the broader question of "how do I make my protein fluoresce?" The advances made in fluorescent protein technology [i.e. principally derivatives of GFP; (Nagai et al. 2002)] mean that the overwhelming majority of the studies summarised below use this ca. 240 amino acid long β-barrel as the fluorescent tag. However, there are alternatives; for example the ca. 180 amino acid SNAP and CLIP tags, derivatives of the O^6-alkylguanine-DNA alkyltransferase, enable fluorescent labelling of proteins in live cells only upon the addition of substrate analogues which covalently bind to the SNAP protein active site (Keppler et al. 2003). The 330 amino acid Halotag system offers many of the same opportunities for fluorescent substrate labelling (Los et al. 2008). These common manipulations come with the inescapable limitation of studying a fusion protein modified with a relatively large tag. Significant efforts have been focussed on new technologies that

enable site-specific fluorescent labelling through less intrusive modification of the target, and we explore these in more detail in the Perspectives section.

Regardless of tag identity, their position in the resultant fusion protein needs consideration and subsequent validation. For example, we and others have observed that N-terminal tagging of ABCG2 with fluorescent proteins is not detrimental to protein localisation or to protein function (Haider et al. 2011; Ni et al. 2010), whereas C-terminal tagging results in the mislocalisation of ABCG2 to intracellular compartments (Haider et al. 2011). Clearly, if the fluorescent tag interferes with protein localisation then subsequent analysis needs to be interpreted with great caution. If the localisation is not impaired by the tagging of the protein, then function should also be investigated to ensure that this is maintained. A final consideration—explored in more detail below—is the expression level of the resultant protein; oftentimes the highest level of expression may be required for structural biology, but for some of the techniques below a "lowest detectable" expression level may be required, for optimal data collection and analysis.

Studies of Intermolecular Interaction and Oligomerisation Using Fluorescence Techniques

In the past decade, several fluorescent techniques have emerged as powerful ways to investigate protein–protein interactions. Overall, three main strategies have been employed to determine the interactions of ABC transporters with other proteins or the oligomeric formation of ABC transporters themselves. The theories of these techniques (FRET, BiFC and TIRF) are summarised in accompanying figures and compared in Table 1.

Fluorescence Resonance Energy Transfer (FRET)

FRET is the most well-known fluorescent technique for the study of protein–protein interaction. The proteins of interest are tagged with two different fluorophores, the donor and the acceptor. Interactions can then be quantified as the energy transfer (FRET efficiency) from the donor to the acceptor, which is measured as the change in donor or acceptor emission (see Fig. 1). To date, this technique has been primarily employed to investigate the protein–protein interactions of several mammalian ABC transporters. Perhaps, the ABC transporters that have been most extensively studied using FRET microscopy are the sulphonylurea receptors (SUR1 and SUR2A), which form part of the K_{ATP} channel with the Kir6.1 or Kir6.2 ion channels. A domain organisation study of SUR1 and Kir6.2 suggested that C-termini of Kir6.2 are centrally located in the K_{ATP} channel and are closer to the NBD1 of SUR1 than the N-terminus of Kir6.2, based on distances determined by

Table 1 Comparison of fluorescence techniques described in this chapter

	FRET	BiFC	TIRF
Live cell application	Yes	Yes	Yes, with particle tracking
Final measurements	Changes in fluorescence signal	Increase in fluorescence signal	Number of photobleaching steps
Distance constraints	Yes	Yes	No[a]
Influence of tag orientations	Yes	No	No
Single wavelength detection	No	Yes	Yes
Complexity of analysis	Medium	Low	High
Real-time detection—e.g. of ligand-induced changes	Yes	No	No
Spatial localisation	Yes	Yes	Plasma membrane only
Controls for non-specific interactions	Yes	Yes	No
Requirement for known oligomer controls	Yes	Yes	Yes
Co-expression of multiple constructs required	Yes	Yes	No
Importance of relative expression levels	High	High	High
Reversibility	Yes (without photobleaching)	No	No
Detection of dimers	Yes	Yes	Yes
Identification of higher order oligomeric state	No[b]	No[b]	Yes

[a]The particle spot size is governed by the characteristics of the TIRF system, limited by the Airy diffraction limit ($\sim$100 nm for green light)
[b]FRET and BiFC have been combined to detect trimeric interactions between partners

relative FRET efficiencies (Wang et al. 2013). FRET has also investigated the proposed interaction of SUR1 with Trpm4 (transient receptor potential melastatin 4). This interaction was not detected when Trpm4 was fused at its C-terminus to the fluorescent protein (Sala-Rabanal et al. 2012). FRET was observed when N-terminally tagged Trpm4 was employed, with the interacting SUR1 and Trpm4 localised to the plasma membrane (see Fig. 1c, d and Woo et al. 2013). Expression level differences and tag localisation have both been proposed to explain the differences in these studies (Woo et al. 2013), highlighting the need for careful construct characterisation before FRET analysis is performed. Gaisano's lab extensively investigated the effect of syntaxin1A (a core member of SNARE protein complex involved in exocytosis) on the K_{ATP} channel, through its interactions with SUR1 or SUR2A using FRET microscopy. Syntaxin1A was shown to negatively regulate the K_{ATP} channel by binding to the NBDs of SUR1 and SUR2A, through

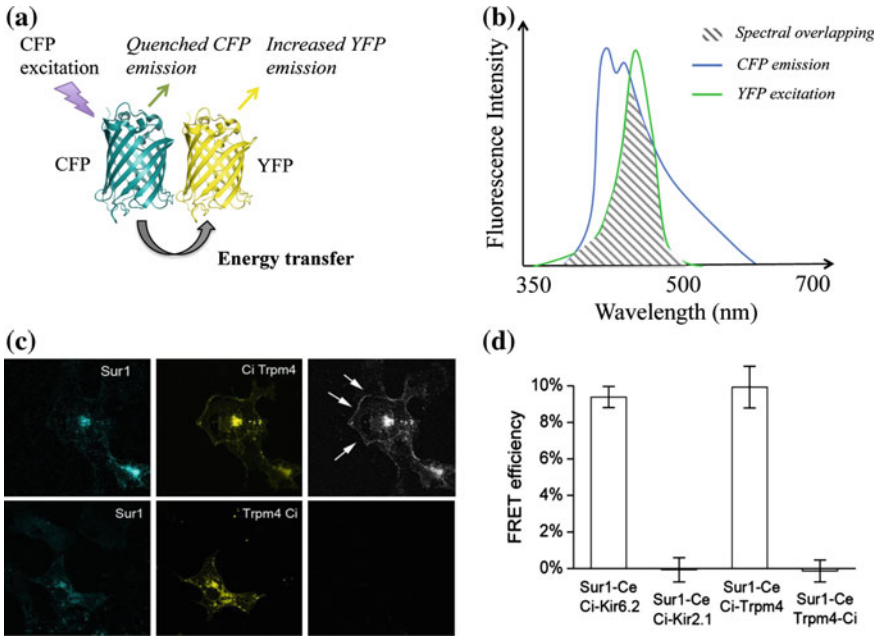

Fig. 1 Fluorescence resonance energy transfer (FRET) (**a**) FRET is a distance-dependent energy transfer process from an excited fluorophore (the donor, here CFP) to another fluorophore (the acceptor, here YFP), which can occur when the emission spectrum of the donor overlaps with the excitation spectrum of the acceptor (**b**), and when the donor and acceptor are in close proximity, less than 100 Å. During FRET, there is a reduction in donor emission and increase in acceptor emission, changes which can be measured as the FRET efficiency to demonstrate close interactions between the fluorescently tagged proteins (**c**). FRET was used to demonstrate the specificity of interaction between SUR1/ABCC8 and Trpm4. Efficient FRET was observed when Cerulean fluorescent protein tagged SUR1 was co-expressed with Trpm4 tagged at the N-terminus with citrine fluorescent protein (*upper panel*, *right*). Tagging Trpm4 at the C-terminus results in no FRET signal (*lower panel*, *right*) (**d**). Quantification of FRET efficiency confirms this specificity. Further FRET experiments confirm that SUR1 interacts with inward rectifier potassium channel 6.2, but not Kir 2.1. Panels (**c**) and (**d**) are taken from Woo et al. (2013) and used with permission of the publisher

in-depth analysis of the differences in FRET efficiency obtained from the various truncated NBD segments with syntaxin1A (Chang et al. 2011; Chao et al. 2011). The same laboratory also identified that ATP regulates the K_{ATP} channel activity in the pancreatic β-cell by modulation of syntaxin 1A interactions with SUR1 using a similar FRET approach (Kang et al. 2011).

Heteromeric organisation of other ABC transporters has also been studied using FRET. Association of P-gp (ABCB1) and CD19 (a lymphocyte antigen) in a B-lymphoma cell line was successfully demonstrated using FRET between fluorophore-conjugated antibodies. This supported a therapeutic strategy to reduce chemoresistance, by P-gp inactivation, using an anti-CD19 antibody to mediate its translocation out of lipid rafts in lymphoma cells (Ghetie 2004). The association of

the cystic fibrosis transmembrane regulator (CFTR, ABCC7) with the epithelial sodium channel (ENaC) was confirmed by Berdiev et al. using FRET microscopy, suggesting that ENaC could have a role in cystic fibrosis pathology (Berdiev et al. 2007).

In addition to the study of heteromeric interactions, FRET has also been employed to study the homomeric interactions in ABC transporters to demonstrate their oligomeric organisation. A dimeric ABCA1 complex formation was shown to be required for function using cells expressing CFP- and YFP-tagged ABCA1 (Trompier et al. 2006). The oligomerisation of several "half-transporters" [i.e. containing only a single NBD and single transmembrane domain (TMD)] has also been studied using FRET microscopy. Hillebrand et al. (2007) showed that the peroxisomal ABC transporters, adrenoleukodystrophy protein (ALDP or ABCD1), formed homodimers and heterodimers with the peroxisome membrane protein 70 (PMP70 or ABCD3) in a FRET study using CFP- and YFP-labelled constructs. This study provided additional evidence that heterodimer formation in ABCD transporters is crucial for the transport of different fatty acids, and contributed to further understanding of the X-linked adrenoleukodystrophy disease (Hillebrand et al. 2007). The oligomerisation of ABCG2, another "half-transporter", was also demonstrated by FRET using CFP- and YFP tagging of ABCG2 in fixed cells (Ni et al. 2010). In bacteria, a 'pixel-level' FRET study showed that a hetero-octameric model is the likely functional unit for the Wzm–Wzt complex, an ABC transporter identified in the *Pseudomonas aeruginosa* for the translocation of the A-band lipopolysaccharides from the cytoplasm to the periplasm. In this study, the authors analysed the histogram distributions of FRET efficiency values obtained in each pixel and reported that the different values obtained corresponded to different oligomeric states. Analysis of the distributions of using simulations based on multiple Gaussian models provided evidence for homo-tetrameric organisation for Wzm and Wzt, and hetero-octameric complexes on their co-expression (Singh et al. 2013).

Bimolecular Fluorescence Complementation (BiFC)

BiFC is based on the refolding of a fluorescent complex when two non-fluorescent complementary fragments of a fluorescent protein are brought together by interacting partners (Fig. 2; Ghosh et al. 2000; Hu et al. 2002). With suitable comparisons, an increase in BiFC signal implicates close/specific association of the proteins to which the non-fluorescent fragments were fused to (Kerppola 2006). Though this process is irreversible, the ability to readily quantify the interaction and provide spatial resolution (Table 1) has attracted several ABC transporter research groups to investigate its applicability to their particular question.

The half-transporters of the ABCG family have been investigated in three studies (Zhang et al. 2010; Haider et al. 2011; McFarlane et al. 2010). In plants [which may be particularly amenable to BiFC studies because refolding works better at lower temperature (Kerppola 2006)], BiFC was used to probe homo- or heteromeric interactions

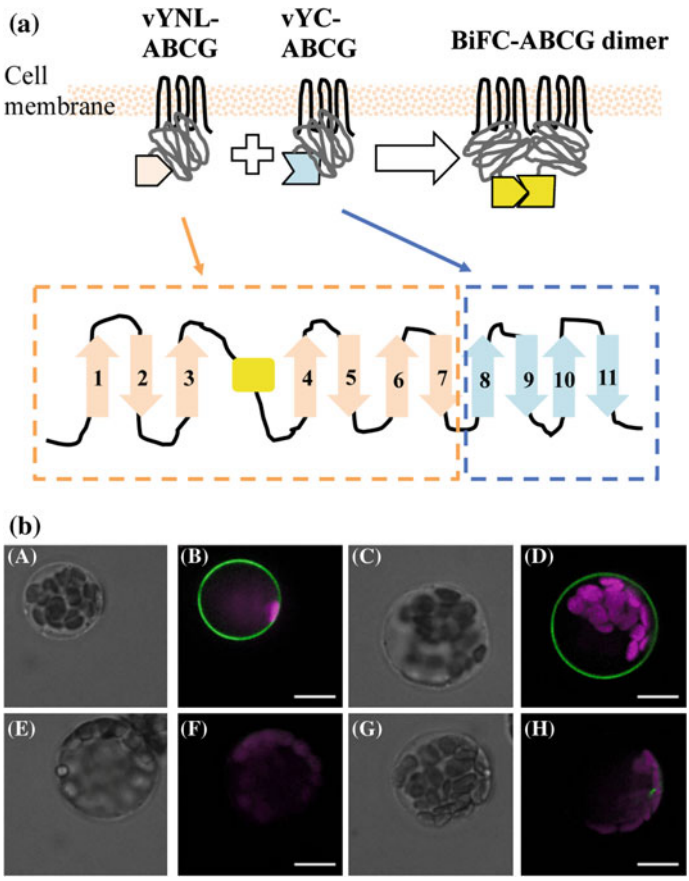

Fig. 2 Bimolecular fluorescence complementation (BiFC). **a** The folding of YFP to form a fluorescent mature protein can be effected by the interaction and assembly of two non-fluorescent fragments of its primary structure, a phenomenon known as bimolecular fluorescent complementation (BiFC) (Kerppola 2006, 2008). The N- and C-terminal fragments of YFP employed (typically residues 1–155 or 1–173 represent the N-terminal fragment, vYNL in the figure, and 155–238 the C-terminal fragment, vYC) are presumed to be unfolded prior to their interaction as they comprise discontinuous elements of the native β-barrel (lower panel). Subsequent to their interaction, the two fragments are essentially inseparable due to the complex intertwined protein interaction interface formed, enabling BiFC to detect and capture potentially transient interactions (Kerppola 2006, 2008). **b** Demonstration of plant ABCG transporter homo- and hetero-oligomerisation. ABCG11 shows membrane-localised BiFC, indicative of homo-oligomeric interactions (image *A* bright field, *B* fluorescence), as well as hetero-oligomeric interactions with ABCG12 (*C, D*). No BiFC is observed with ABCG12 alone (*E, F*) indicating this transporter does not homo-oligomerise. Specificity of the interactions is confirmed by a lack of BiFC between ABCG11 and an unrelated nitrate transporter NRT3 (*G, H*). Data are republished from McFarlane et al. (2010) with permission of the publisher

in the cuticular lipid transporters ABCG11 and ABCG12. Co-transfection into plant protoplasts provided evidence for specific heterodimers of ABCG11 and ABCG12, and for homodimers of ABCG11, but not for ABCG12 homodimers. McFarlane et al. controlled for non-specific complementation by demonstrating the absence of BiFC on co-transfection of single ABCG11/12 constructs with an unrelated nitrate transporter partner (see Fig. 2b and McFarlane et al. 2010). The functional significance of heterodimerisation was confirmed by the analysis of knock-out mutants of both transporters (McFarlane et al. 2010). Heterodimeric, rather than homodimeric, interaction of plant ABCG transporters was also shown in *Medicago* for two novel members of the ABCG family associated with vascular plants, and essential for the structural hyphae that are required for symbiosis with mycorrhizal fungi (Zhang et al. 2010). The STR and STR2 were co-localised to these structures and showed BiFC only when co-expressed (Zhang et al. 2010). In the ABCG family, human ABCG2 homodimerisation was visualised in the plasma membrane of cultured HEK293T cells (Haider et al. 2011), whereas non-specific interaction with a G-protein coupled receptor retained the BiFC complexes intracellularly. Quantitative analysis of fluorescence complementation of wild-type and mutant ABCG2 isoforms demonstrated that the stability of the ABCG2 homodimer is insensitive to an intermolecular disulphide bond (Haider et al. 2011; Kage et al. 2005).

A further use of BiFC has been to confirm the interaction between an NBD (STAR1) and a TMD (STAR2) of a rice ABC transporter implicated in aluminium tolerance (Huang et al. 2009). BiFC was used in rice protoplasts to confirm the predicted intermolecular interaction and the localisation of the BiFC signal to intracellular vesicles, believed to be mechanistically important in aluminium tolerance (Huang et al. 2009). Finally, BiFC has also been employed as a follow-up to confirm the results of genomewide interaction mapping using the split ubiquitin system (Snider et al. 2013). The authors employed the split ubiquitin system to identify the interactome of 19 *S. cerevisiae* ABC transporters and subsequently employed BiFC to validate 79 interactions involving 17 ABC transporters. Just over half the interactions (56 %) were validated by BiFC, presumably reflecting more stringent requirements in the BiFC assay compared to the original screen (Snider et al. 2013).

Total Internal Reflection Fluorescence (TIRF)

Single particle imaging TIRF microscopy is an advantageous illumination technique as it produces a thin layer of excitation of the sample just above the coverslip (see Fig. 3), which offers improved resolution depth (typically 100 nm or less) of labelled plasma membrane proteins. Identification of single fluorescent spots is thus made possible, and at suitably low expression levels these spots may be assumed to derive from “single” complexes containing the fluorescent labelled protein. Under these conditions photobleaching with high power laser will result in the reduction of fluorescent intensity of the fluorescent spot in a stepwise manner (Fig. 3b and Nagata et al. 2013), revealing the oligomeric state of the protein of interest. The

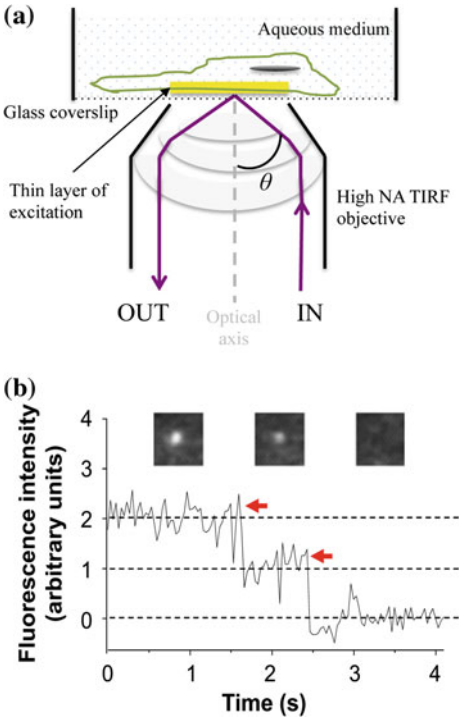

Fig. 3 Total internal fluorescence reflection (TIRF). **a** The TIR principle is derived from Snell's law where light propagates through a medium of high refractive index (glass) to another medium of low refractive index (aqueous), the angle of subsequent refracted beam is affected by the angle of incidence (θ). As θ becomes larger and exceeds a 'critical angle', the excitation light is reflected back into the medium of high refractive index (the glass). This generates an electromagnetic field in the aqueous medium adjacent to the plane of incidence, known as the evanescent field. The intensity of this evanescent field decreases exponentially as it propagates into the aqueous medium, generating a thin layer of excitation at the glass coverslip surface (Axelrod 2001). In TIRF microscopy, this large angle of incidence is achieved using a high numerical aperture objective >1.45 (higher than the refractive index of water, 1.33) and by positioning the excitation laser away from the optical axis at the back focal plane. **b** TIRF microscopy images of ABCA1 tagged with monomeric EFGP show stepwise photobleaching intervals consistent with ABCA1 dimers. Fluorescence images are shown at three representative points in the stepwise photobleaching time course. Data in panel b are taken from Nagata et al. (2013) with permission of the publisher

oligomeric formation of three human ABC transporters was investigated using this technique. Haggie and Verkman first employed TIRF microscopy to achieve single molecule imaging of GFP-tagged CFTR. Complementary analysis of intensity distributions and number of photobleaching steps, in comparison to purified monomeric GFP, suggested that CFTR is most likely to exist as monomers in the plasma membrane (Haggie and Verkman 2008). Using TIRF stepwise photobleaching methods, Nagata et al. (2013) showed that ABCA1 is likely to exist as monomers and dimers, instead of dimers and tetramers as suggested by Trompier

et al. (2006) (using FRET microscopy as mentioned above). Particle tracking, combined with stepwise photobleaching analysis, enabled Nagata et al. to derive a novel model for the mechanism of ABCA1 during HDL generation whereby ABCA1 dimers dissociate into monomers upon binding apolipoprotein A1 (Fig. 3b and Nagata et al. 2013). The contradictory evidence presented by Nagata et al. compared to Trompier et al. (2006) was attributed to the differences in methodologies (single particle fluorescence imaging vs. ensemble fluorescence measurements) employed and possibly even differences in expression levels of ABCA1 in the cells (Nagata et al. 2013). Recently, the oligomerisation of ABCG2 has been examined in fixed cells using a combination of TIRF and automated detection and analysis of fluorescent spot photobleaching steps (Wong et al. 2015) allowing the authors to propose a predominant tetrameric state for this transporter.

Solution-Based Intramolecular FRET Determinations of ABC Transporter Structure and Mechanism

The FRET studies discussed above all use confocal microscopy to determine energy resonance and infer spatial proximity. A number of other studies on ABC transporter have used purified, detergent solubilised proteins to carry out solution-based ensemble FRET measurements, and these are considered briefly here. Several papers from the Sharom group have shown that FRET can provide spatial data on the ATP-binding pockets and the drug-binding sites on the TMDs of P-gp (Liu and Sharom 1997; Lugo and Sharom 2005; Qu and Sharom 2001, 2002). These studies have taken advantage of cysteine resides in the Walker-A motifs of P-gp, allowing labelling of the NBDs with one or two fluorophores, and the availability of fluorescent drug molecules to label the drug-binding sites. Distinct labelling of the two NBDs was used to propose that the Walker-A motifs were 15–20 Å apart, consistent with determinations of NBD-dimer crystal structures that were becoming available (Qu and Sharom 2001), whilst similar strategies enabled spatial estimates to be made between the Walker-A motifs and the "R" and "H" drug-binding sites on the TMDs (Lugo and Sharom 2005; Qu and Sharom 2002). Spatial mapping approaches have also been used to explore the NBDs and TMDs of the osmoregulatory ABC transporter OpuA (Gul et al. 2012).

In a well-controlled study that placed significant emphasis on separating unlabelled protein from fluorophore-labelled proteins, Horn et al. spatially quantified the monomer–dimer equilibrium of the two NBDs of an OpuAA dimer upon transition through the catalytic cycle. These distances could be reconciled with the X-ray structural data on periplasmic binding protein-dependent ABC transporters (Horn et al. 2008). Other domain–domain interaction studies have employed FRET to examine the interaction between components of energy coupling factor-type ABC transporters [where domain stoichiometry was also investigated (Finkenwirth et al. 2010)], between TAP1 and TAP2 (Geng et al. 2013), and between homologous halves of fluorophore-labelled single cysteine isoforms of P-gp (Verhalen et al.

2012). For the latter two studies the proximity of the two halves of an intact ABC transporter in the presence or absence of nucleotide or transport substrate was examined. Ensemble FRET ratios argued for nucleotide-dependent closer interactions of the two halves of the transporter (Geng et al. 2013; Verhalen et al. 2012). For P-gp, the same group of authors (Verhalen et al. 2012) developed a novel high-resolution spectroscopy method (10 fL detection volume) to measure the FRET efficiency and estimate the distances between the two NBDs of P-gp, using a Hidden Markov model in combination with dwell time determinations to interpret the data in terms of multiple conformational transitions (Zarrabi et al. 2014). Comparisons of FRET data in the presence or absence of transport substrates have added further weight to the proposal that ABC exporters will not share a common mechanism (Geng et al. 2013; Verhalen et al. 2012). More recently, a dual-colour, functional ABCC1 has been developed to allow for studies of NBD closure (increased FRET) following nucleotide and/or transport substrate addition. The authors demonstrated that the construct could enable a high throughput screen of potential ABCC1 inhibitors (Iram et al. 2015). Most recently, an incredibly elegant, single molecule FRET study enabled Thorben Cordes and colleagues to present a mechanism for substrate capture and release within the ABC importer GlnPQ, linked to the structural dynamics of its substrate binding domains (Gouridis et al. 2015).

FRAP Diffusion Measurements Allow Cellular Microenvironment Predictions

Single cell diffusion studies of fluorescently labelled proteins in live cells using fluorescence recovery after photobleaching (FRAP) microscopy are commonly employed to estimate the diffusion of target proteins of interest, allowing the prediction of their behaviour within the respective microenvironments (Lippincott-Schwartz et al. 2001). In this technique, the recovery of neighbouring fluorescent molecules into a photobleached region is monitored over time and the subsequent fluorescence recovery curve is used to determine the diffusion parameters by fitting the curve with an exponential equation (see Fig. 4). Despite being a powerful technique for many membrane proteins, only a handful of ABC transporters have been characterised by FRAP microscopy thus far.

The mobility and organisation of TAP1 in association with its transport function (translocation of peptides from cytosol into endoplasmic reticulum for immune response of the cell) was examined using FRAP microscopy by Neefjes's group (Reits et al. 2000). The diffusion rate of TAP1 (tagged with GFP), which correlates with TAP transport activity, decreased during translocation (i.e. increased peptide substrate concentration), consistent with TAP recruitment to a peptide loading complex (Reits et al. 2000; Wearsch and Cresswell 2008). Analysis of reduction in diffusion rates of peptide-saturated cells compared to peptide-free cells suggested that about one-third of the TAP molecules are constitutively active, positing a role for TAP in the continuous surface presentation of peptides from nascent proteins

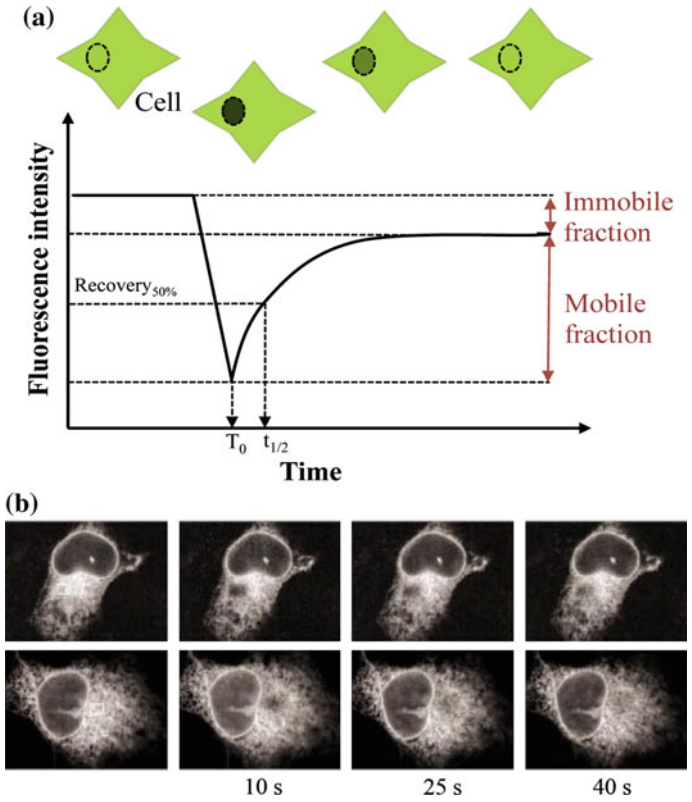

Fig. 4 Fluorescence recovery after photobleaching (FRAP). **a** FRAP is a technique to measure the diffusion during the exchange of neighbouring non-photobleached fluorescent molecules into a region with photobleached molecules. In FRAP experiments, the background fluorescence intensity is first recorded over a short period time, before the selected region of the cell is photobleached by high laser power and the subsequent increase in fluorescence intensity is monitored over a period of time, using normal acquisition laser power. The recovery of the fluorescence intensity can then be modelled using exponential equations to estimate the half time ($t_{1/2}$) and the mobile fractions of the fluorescent proteins of interest, which indicate the time taken to achieve 50 % recovery and the fraction of fluorescent molecules that contributed to the recovery (Lippincott-Schwartz et al. 2001). **b** Demonstration of TAP1/TAP2 interactions using FRAP microscopy. *Upper panel* shows slow FRAP recovery of TAP1-GFP in the presence of a full-length TAP2, indicative of complex formation between TAP1 and TAP2. FRAP recovery is much faster in the presence of a truncated TAP2 construct (*lower panel*) demonstrating the regions of TAP2 interacting with TAP1. Data in (**b**) are taken from Vos et al. (2000) with the permission of the publisher

(Reits et al. 2000; Wearsch and Cresswell 2008). Another FRAP study on the mobility of TAP complexes using truncated TAP1 (transmembrane helices 2-6, TM2-6) and TAP2 (TM1-4) mutants and full-length TAP1 (TM1-6) and TAP2 (TM1-5) showed that the TM1 of TAP1 and the TM5 of TAP2 were important for the formation and function of TAP complex, as the truncated mutants did not reduce the mobility TAP when co-transfected with the full-length TAP1 or TAP2

(see Fig. 4b and Vos et al. 2000). These results provided evidence for alignment of TAP1 and TAP2 in a head-to-head and tail-to-tail orientation within the TAP complex (Vos et al. 2000).

The diffusion of CFTR was also investigated using FRAP microscopy. Upon investigating the GFP-tagged dysfunctional CFTR mutant (ΔF508) using FRAP microscopy, Haggie et al. showed that the ER-retained CFTR mutant did not have restricted mobility, and was not aggregated or bound to slow-moving membrane proteins. However, FRAP provided evidence that the mutant CFTR interacts more strongly with ER chaperones than the wild-type protein (Haggie et al. 2002). Two independent FRAP studies on CFTR demonstrated the role of C-terminal PDZ-binding motif in limiting membrane diffusion of CFTR through transient interactions with the cell cytoskeleton (e.g. actin) (Bates et al. 2006; Haggie et al. 2004). As the lateral mobility of CFTR was poorly understood, the importance of PDZ domain and the general dynamics of CFTR in ER or plasma membrane revealed in these studies may be used to provide further information on the regulation of this ion channel in the future.

In plants, FRAP analysis demonstrated that the auxin efflux transporter, ABCB4, formed stable association at the plasma membrane and it had distinct endocytic properties compared to the PIN-FORMED protein, suggesting that ABCB4 is a constitutive auxin exporter (Cho et al. 2012). The examples of studies shown here suggest that the diffusion properties, the molecular association and the cellular microenvironment of ABC transporters can be predicted using FRAP microscopy. In addition to monitoring the FRAP of the ABC transporter itself, studies elsewhere also provided further understanding of the ABCA1 mediated cholesterol efflux by monitoring the FRAP of phosphatidylcholine in the membrane (Zarubica et al. 2009).

Future Perspectives

These studies illustrate how fluorescence microscopy provides a powerful driver to explore ABC transporter dynamics and function (Table 1). The convenience of fluorescent protein tagging, coupled with the attraction of analysis in intact or living cells, makes such methods a vital element in probing intramolecular conformation changes, oligomerisation and protein–protein interactions. In the excitement of what such investigations tell us about these processes, their limitations are sometimes forgotten. The necessity in nearly all cases to study highly modified proteins, in a recombinant cell environment, requires careful consideration and appropriate controls. How might transporter complex composition, or its spatial localisation and dynamics, be affected by fusion proteins at high expression levels in a non-native cellular context? Second, many of the fundamental questions we ask—how do transporter proteins work or interact with one another to control function—really need visualisation of interactions at the molecular level. It is important to realise that the FRET, BiFC and TIRF strategies described above all infer these indirectly. For standard fluorescence microscopy methods, there are constraints based on light

diffraction (Abbé's limit) which fundamentally limit spatial resolution to around 100 nm, at least 10-fold greater than the diameter of a single transporter protein.

The fast-moving microscopy field suggests that potential solutions to these problems will become within reach of most biological researchers within a few years. Protein engineering strategies are emerging that will avoid the detractions of a large fluorescent protein tag. These include coenzyme A based systems [involving enzyme transfer of the fluorophore to a tag which can be as small as 11 amino acids; (George et al. 2004; Yin et al. 2005)], biarsenical labelling of tetracysteine recognition sequences (Adams and Tsien 2008), and perhaps most promisingly, the incorporation of single fluorescently tagged unnatural amino acids at the desired position (Zhang et al. 2013). In the past, transgenic strategies have allowed GFP-tagged proteins to be expressed in vivo in native context and under transcription control, including CFTR (Oceandy et al. 2003). The goal of endogenous protein labelling in a selective manner, and its sensitive fluorescent detection at low expression levels, is a little further afield. However, ligand-directed tosyl chemistry shows at least one route by which this might be achieved (Tsukiji and Hamachi 2014), bearing in mind the significant effort of generating selective delivery ligands for the transporter of interest. Such advances coincide with developments in the resolution of intravital microscopy, in which multiphoton techniques, often combined with far red fluorophores, can overcome light absorption and scattering problems associated with imaging at depth in tissues. It is not unrealistic to await imaging and FRET studies on transporters in situ, within organs or tumours of living animals (Ellenbroek and van Rheenen 2014).

What of the "unbreakable" Abbé's limit, and the desire to image at true single molecule resolution? There are methods already available (such as fluorescence correlation spectroscopy to measure particle mobility and photon counting histogram analysis to define molecular brightness) that enable, at least statistically, determination of single fluorescent molecule's behaviour and composition within a small region ($\sim$0.1 μm^2) illuminated by the confocal volume (Weidemann et al. 2014). Indeed, we have recently published a combined TIRF and fluorescence correlation spectroscopy analysis of ABCG2 that demonstrates a predominantly tetrameric organisation of this transporter (Wong et al. 2015). Correlative approaches can combine the dynamic potential of fluorescence light microscopy with the resolving power of cryo-electron tomography in the same carefully mapped specimens (Zhang 2013). Most excitingly, the rise of "super-resolution" microscopy techniques has been meteoric in the past two decades, from a futile fight against what seemed a fundamental law of physics, to the award of the 2014 Nobel Prize in Chemistry and commercial microscope systems on the market. A range of approaches provide spatial resolving power at the nm scale (see Godin et al. (2014) for a review), bringing direct studies of single molecule organisation within reach. Moreover, some of these methods (such as photo-activatable light microscopy, PALM) can be implemented with readily available fluorophores and fluorescent proteins, and retain the essential advantage of investigating the dynamic environments of living cells, opening up new avenues for studying ABC transporters at the single molecule level.

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Part II
Human ABC Transporters of Medical Relevance

Genetic Polymorphisms of P-glycoprotein: Echoes of Silence

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Abstract ABCB1 is a polymorphic gene that encodes a full ABC transporter with drug-binding pockets and two nucleotide-binding ATPase domains. It was one of the first members of the ABC transporter superfamily to be identified in humans. Since the discovery of this gene nearly 40 years ago, many mutations and polymorphisms in the coding region have been shown to have functional significance. Common genetic variations in the form of single nucleotide polymorphisms (SNPs) of ABCB1 have been shown to have a role in disease susceptibility and drug response. In this chapter, we summarize our current understanding of common ABCB1 SNPs and their impact on protein folding, drug transport function, disease risk factors, and drug pharmacokinetics. Unfortunately, clinical studies on the association of drug effects and ABCB1 polymorphisms are often inconclusive. In the past few years, meta-analyses of ABCB1 polymorphism studies have been carried out in attempts to draw more consistent conclusions. Among ABCB1 SNPs, the synonymous C3435T polymorphic site is perhaps one of the best-known silent mutations in the field of pharmacogenomics. Our current understanding of the mechanism of the effect of this "silent" SNP is discussed here. Overall, research efforts in the past 15 years have laid important groundwork concerning the effect of ABCB1 variants that should lead to advances in precision medicine in the future.

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Introduction

The ATP-binding cassette (ABC) membrane transporter superfamily includes integral membrane proteins that serve a wide range of important cellular and biochemical functions. It represents the largest family of trans-membrane proteins. ABC transporters are found in prokaryotes as well as eukaryotes, suggesting their importance for life and evolution. The eukaryotic ABC transporters share two common features: each protein contains a highly conserved nucleotide-binding domain (NBD) and trans-membrane domain (TMD). The NBD binds ATP and uses the energy produced from ATP hydrolysis to drive the substrate of the transporter across the cell membrane (Higgins 1992). In humans, there are 48 identified ABC transporter genes that have been divided into 7 subfamilies (A to G) based on sequence homology, and are found to be differentially expressed in different organs (Dean et al. 2001). All human and mouse ABC transporter gene names were standardized by the Human Genome Organization (HUGO) in October 1999 (http://www.genenames.org/genefamily/abc.html). In this chapter, we will focus on the human ABC transporter ABCB1 (P-gp/MDR1/PGY1), which is one of the best-characterized efflux transporters.

General Features of ABCB1

The ABCB1 transporter was first described in tumor cells that had acquired resistance to anticancer agents (Riordan et al. 1985; Akiyama et al. 1985; Kartner et al. 1983a, b). In 1976, Juliano and Ling published a paper in Biochimica et Biophysica Acta that identified P-glycoprotein (P standing for permeability) as linked to the multidrug resistance (MDR) phenotype observed in Chinese hamster ovary cells (Juliano and Ling 1976). However, the gene was not successfully cloned from drug-resistant KB carcinoma cells until 10 years later (Roninson et al. 1986). As of early 2015, nearly 20,000 scientific articles related to this gene can be found in PubMed using the keyword "ABCB1", and 20,000 more using the original name "P-glycoprotein". The ABCB1 gene is found on chromosome 7, at band p21-21.1 (Chen et al. 1990). Its location is very close to another B family member, ABCB4. *ABCB1* spans approximately 209.6 kb, with 28 exons ranging in size from 49 to 587 bp (Raymond and Gros 1989). The corresponding messenger RNA (mRNA) is 4872 bp in length, including the 5′ untranslated region (http://www.ncbi.nlm.nih.gov/gene/5243). The ABCB1 gene encodes a polypeptide with 1280 amino acids which folds into six hydrophobic trans-membrane domains, and two NBDs. In SDS–PAGE, P-gp has an apparent molecular weight of 170 kDa. The two halves are separated by a flexible linker region, and the two ATP-binding domains are structurally similar. There are 12 trans-membrane domains. The mature protein is N-glycosylated, so the oligosaccharide group is sensitive to PNGase F but not Endo H treatment (Richert et al. 1988). There are also several phosphorylation sites

in the linker region. However, neither the N-glycosylation nor any of the phosphorylation sites plays an essential role in the establishment of the multidrug resistance phenotype (Germann et al. 1996; Gribar et al. 2000). Several motifs have been identified in each of the ATP-binding domains, including the Walker-A (Mizutani and Hattori 2005), Walker-B (Mizutani and Hattori 2005), A-loop (Ambudkar et al. 2006), H-loop (Gottesman et al. 1996), D-loop (Gottesman et al. 1996), Q-loop (Mizutani and Hattori 2005), and the signature motif containing the "LSSGQ" consensus sequences (Loo et al. 2002). The ATP-binding domains act as ATPases that hydrolyze ATP to ADP (Hrycyna et al. 1998).

Tissue Distribution and Function of ABCB1

P-gp is present in the brain (the capillary endothelial cells), small and large intestinal epithelium, kidney, liver (the canalicular membrane of the hepatocytes), pancreas (pancreatic ductile cells), and the adrenal gland (Fojo et al. 1987; Sugawara et al. 1988; Thiebaut et al. 1987), and during pregnancy, P-gp is expressed in the trophoblast layer of the placenta (Nakamura et al. 1997; Mylona et al. 1996). In polarized epithelia, P-gp is expressed on the apical membrane, facilitating transport in a directional manner (Thiebaut et al. 1987; Horio et al. 1989). Loss of ABCB1 is not lethal (Schinkel et al. 1997). However, deletion mutations in the P-gp gene of certain collies have been shown to render the animals hypersensitive to ivermectin (Mealey et al. 2001). Genetic knockout studies have shown that mice with the MDR1a/b gene knocked out have altered tissue distributions of drugs such as digoxin (Schinkel et al. 1995). The loss of this gene also affects elimination from the body of a broad spectrum of cationic amphiphilic drugs via the liver and intestine (Smit et al. 1998). The occurrence of P-gp in normal tissues and its localization suggests its primary physiological function is to protect organs from damage caused by xenobiotics.

Substrate Specificity of ABCB1

A unique feature of P-gp is its broad substrate specificity, ranging from small molecules such as organic cations, carbohydrates, amino acids, and some antibiotics to macromolecules such as polysaccharides and proteins (Zhou 2008). Most of these substrates are weakly amphipathic and relatively hydrophobic, often containing aromatic rings and a positively charged nitrogen atom (Sharom 2011). It has been suggested that the only common property among P-gp substrates is their relatively hydrophobic and amphiphilic nature (Gottesman and Pastan 1993). The list of ABCB1 substrates continues to grow (a list can be found in references Silva et al. 2014; Cascorbi and Haenisch 2010; Fromm 2004). Interestingly, many substrates of ABCB1 have also been identified as substrates of cytochrome P450,

which has no obvious sequence or structural homology to ABCB1. A substrate enters the substrate-binding site, which is formed by the TMDs. Unfortunately, how compounds interact with the substrate-binding site in the drug-binding pocket is still unclear. Recent studies by site-directed mutagenesis indicate the existence of multiple transport-active binding sites in P-gp for a single substrate. Thus, drugs can bind at either primary or secondary sites (Chufan et al. 2013; Chufan et al. 2015).

Polymorphisms of ABCB1

Genetic Mutations Influence P-gp Function

In the past 20 years, studies have indicated that the function of ABCB1 is greatly influenced by genetic mutations. Site-directed mutagenesis studies have demonstrated that ABCB1 function, including drug efflux and ATPase activity, can be affected by mutations in the substrate-binding pocket or ATP-binding domains. For example, mutations in the Walker-A (Kim et al. 2006) or Walker-B (Sauna et al. 2007) domains in either of the ATP-binding domains render the ABCB1 protein unable to efflux its substrates. Also, mutations in the trans-membrane domains can alter substrate-binding affinity (Loo et al. 2006; Loo and Clarke 1993a, b; Hafkemeyer et al. 1998, G2677T/A). In 1994, Stein et al. were the first to report the existence of point mutations at +103 and +137 in the ABCB1 promoter (Stein et al. 1994). Since then, there has been a great deal of interest in testing the effects of genetic mutations in the human ABCB1 gene region. Genetic variations, in the form of single nucleotide polymorphisms (SNPs), by definition are common, but minute variations that occur in the human genome at a frequency of 1 every 1000 bases. They are frequently found in the human ABCB1 gene region. In the coding region of ABCB1, the first SNP was reported in 1998, when Mickley et al. discovered two mutations at G2677T/A (G → T) and at 2995 (G → A) (Mickley et al. 1998). Today, more than 370 SNPs of this gene are listed in the National Center for Biotechnology Information (NCBI) dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?locusId=5243). Therefore, ABCB1 is highly polymorphic. In the year 2000, Hoffmeyer et al. reported a systematic investigation of ABCB1 SNPs and discovered the existence of a SNP in chromosome 26 (rs1045642, C3435T, Ile1145Ile) (Hoffmeyer et al. 2000). In that study, a significant correlation of the SNP with expression levels and function of MDR1 was found, and individuals homozygous for this polymorphism had significantly lower duodenal ABCB1 expression and the highest digoxin plasma levels (Hoffmeyer et al. 2000). In the following years, Kim et al. (2001) and others (Kroetz et al. 2003; Cascorbi et al. 2001) reported direct sequencing results revealing that the C1236T, G2677T/A, and C3435T SNPs are frequently found in human populations and appear in strong linkage disequilibrium. This pilot study triggered a large number of

subsequent investigations on the effect of ABCB1 SNPs. As of 2015, there were more than 1500 published articles listed in PubMed (www.nibi.nlm.nih.gov/pubmed) that mention "polymorphisms" and "ABCB1". Several studies indicated that the ABCB1 C3435T SNP is in strong linkage disequilibrium with the G2677T/A (rs2032582, Ala893Ser/Thr) SNP (Kim et al. 2001; Horinouchi et al. 2002; Tanabe et al. 2001) and with another synonymous SNP, C1236T (rs1128503, Gly412Gly) (Kim et al. 2001). These three SNPs form the most common MDR1 haplotype (Ozawa et al. 2004).

Genetic Variations of ABCB1 in Different Populations

Perhaps the most interesting characteristic of the C3435T SNP is its frequency of occurrence in various populations. A number of studies have addressed this issue by studying the occurrence of the 3435 variant T allele in different populations including Han Chinese, Indians, Turks, Africans, Caucasians, Central Americans, South Americans, Ashkenazi Jews, and New Zealanders, concluding (Fung and Gottesman 2009; Ameyaw et al. 2001; Sipeky et al. 2011; Ieiri 2012; Hubacek 2014; Engen et al. 2006) that the 3435 C allele is most frequently found in African blacks, (Sipeky et al. 2011). However, Egyptian Africans are quite different, with a prevalence from 0.48 to 0.60 (Ebid et al. 2007; Hamdy et al. 2003), which is close to the frequency of this SNP in Caucasians, ranging from 0.43 to 0.62 (Sipeky et al. 2011). In Asians, the frequency of the T allele ranges from 0.46 to 0.67 (Sipeky et al. 2011). This allele is the major allele in Indians, with more than 63 % of the Indian population carrying at least one T allele (Chowbay et al. 2003). Differences in allele distribution in various populations might predict possible interindividual or interethnic differences in drug pharmacokinetics and disease prognosis.

Recent Progress on the Clinical Impact of ABCB1

Discovery of Clinically Relevant ABCB1 Polymorphisms

At the beginning of the twenty first century, there was an increase in research on the clinical and in vitro relevance of variations of ABCB1. This was, in part, due to the discovery of the ABCB1 C3435T SNP by Hoffmeyer and colleagues (Hoffmeyer et al. 2000). In their study, the MDR1 sequence and expression from a small number of volunteers ($n = 21$) were analyzed and a correlation between expression levels and a polymorphism in exon 26 (C3435T) was found. The variant T allele carriers had significantly lower duodenal MDR-1 expression and high digoxin plasma levels. This was the first clinical evidence that a common SNP in human ABCB1 could have a clinical impact. After 15 years of continued research, the pharmacokinetic significance of ABCB1 variants still remains an active area of

study. A major focus has been on correlating ABCB1 polymorphisms with disease or drug treatment response. Given the ability of ABCB1 transporters to efflux a variety of chemotherapeutic agents, cancer therapy represents an important subject to understand the influence of ABCB1 variants, with much investigative attention being directed toward breast cancer, colorectal cancer, lung cancer, acute myeloid leukemia, and chronic lymphocytic leukemia (Table 1). The association of ABCB1 polymorphisms with HIV/AIDS, Alzheimer's disease, Parkinson's disease, depression, and epilepsy has also drawn significant attention. These diseases are directly or indirectly related to drug treatment, as the drugs currently used to treat these diseases interact with P-gp. Imatinib (Shukla et al. 2008), anthracyclines (Pastan et al. 1988), taxanes (Horwitz et al. 1993), and most protease inhibitors (Lee et al. 1998) are known P-gp substrates. The studies most commonly found on PubMed concerning ABCB1 polymorphisms and their effect on drug

Table 1 Polymorphisms of P-gp reported in clinical research articles from 2000 to 2014

Disease	No. of studies	Drug name/group	No. of studies
Epilepsy	80	Tacrolimus	106
Breast cancer	58	Protease inhibitors	57
HIV/AIDS	57	Cyclosporin	54
Colorectal cancer	36	Taxane	49
Inflammatory bowel disease	35	Clopidogrel	47
Lung cancer	25	Statin	32
Acute lymphoblastic leukemia	24	Imatinib	24
Chronic myeloid leukemia	21	Irinotecan	18
Ulcerative colitis	19	Digoxin	16
Acute myeloid leukemia	17	Methadone	15
Depression	16	Opioids	15
Ovarian cancer	16	Anthracycline	13
Parkinson's disease	14	Ivermetin	12
Gastric cancer	12	Morphine	12
Chronic lymphoblastic leukemia	9	Stem cell transplant	11
Lymphoma	8	Carbamazepin	11
Liver cancer	7	Vinblastine/vincristine	8
Glioblastoma	5	Sirolimus	7
Renal cancer	5	Fexofenadine	7
Alzheimer's disease	5	Etoposide	6
Prostate cancer	3	Oxycodone	5
Pancreatic cancer	3	Dexamethasone	4
Gastrointestinal	3	Vinorelbine	4
Not specified	3	Mitoxantrone	2
Colon cancer	2		
Thyroid cancer	1		

pharmacokinetics are related to two immunosuppressors, tacrolimus (FK-506 or fujimycin) and cyclosporin (a total of 157 studies found until March, 2015), which are frequently administered to renal transplant patients. In the last few years, there has been a great deal of interest in studying the effect of clopidogrel (Notarangelo et al. 2013; Ross and Pare 2013), a blood thinner. It is an inactive prodrug that requires active enteric absorption, where it is modulated by intestinal P-gp (Luo et al. 2012). These studies indicate that there exists considerable and sustained interest in investigating possible genetic correlations between ABCB1 SNPs and drug pharmacokinetics.

Pitfalls in ABCB1 Polymorphism Association Studies

Although there has been substantial effort to investigate the polymorphisms of ABCB1, some concerns have been raised. First, most, if not all, of the studies have focused only on the haplotype that includes the C1236T, G2677T/A, and C3435T, while some have analyzed only the C3435T SNP. Since ABCB1 is a polymorphic gene, the potential effect of other important ABCB1 SNPs (e.g., G1199A) might be ignored. Second, some of the clinical studies purporting genotype–phenotype relationships were generated by a very small sample size, which increases the chance of sample bias. Third, very often the entire gene was not sequenced and therefore, additional interactions and the effect of other co-occurring SNPs were not considered. Fourth, some studies only investigated one SNP of the ABCB1 haplotype (based on the assumption of linkage disequilibrium) in a heterogeneous population, in which some subjects carried only one SNP, while others might have two or three of the haplotype SNPs. These factors have all contributed to the conflicting conclusions drawn from clinical studies.

Clinical reports showing contradictory correlations of ABCB1 polymorphisms with drug response have been found in the context of several drugs, including paclitaxel, imatinib, loperamide, nelfinavir (Hirt et al. 2008; Saitoh et al. 2005), methadone (Levran et al. 2008; Coller et al. 2006), and tacrolimus (Macphee et al. 2002; Anglicheau et al. 2003). Investigating whether certain ABCB1 genotypes are associated with sensitivity of cancer to platinum or taxane therapy, one study reported that the G2677T/A and 3435T SNPs were associated with poorer outcomes (Pan et al. 2009), two studies reported variant alleles had better drug responses (Green et al. 2006; Johnatty et al. 2008), and three studies found no associations with either the G2677T/A or C3435T SNPs (Marsh et al. 2007; Peethambaram et al. 2011; Tian et al. 2012). Loperamide is a potent drug used to treat diarrhea that is widely used around the world. It is an opioid and an avid substrate for ABCB1. In the year 2003, two papers were published with opposite results. One study suggested that the 2677G and 3435T haplotype was associated with higher loperamide concentration in plasma (Skarke et al. 2003). Another study found no such correlation in healthy white volunteers (Pauli-Magnus et al. 2003). Imatinib is an anticancer drug that is a tyrosine kinase inhibitor. It is also known as a substrate of ABCB1 (Shukla et al. 2008). In 2006 and 2008, two studies reported

that the pharmacokinetics of this drug and polymorphisms of ABCB1 were not significantly associated (Gardner et al. 2006; Petain et al. 2008). However, an association between ABCB1 and imatinib clearance was demonstrated in another study that observed lower imatinib clearance from day 1 to steady state in wild-type ABCB1 (Gurney et al. 2007). In other work, Yamakawa et al. demonstrated higher imatinib clearance in Japanese chronic myeloid leukemia (CML) patients with the C3435T genotype (Yamakawa et al. 2011). Studies have also indicated that imatinib clearance appears to be more influenced by polymorphisms of ABCG2 (Koo et al. 2015; Di Paolo et al. 2014). These conflicting reports show the complexity of this issue.

Conflicting reports are also found regarding ABCB1 polymorphisms and risk factors for diseases such as epilepsy, Parkinson's disease, cancer, etc. One disease for which there are conflicting reports is inflammatory bowel disease (IBD), a chronic inflammation of all or part of the digestive tract. One study found that the ABCB1 C3435T polymorphism is associated with IBD (Ho et al. 2006), while two studies concluded that there is no association (Dudarewicz et al. 2012; Oostenbrug et al. 2006). Reports with opposite results raise serious questions concerning whether associations of ABCB1 polymorphisms with drug response or disease risk are statistically strong enough to make a reasonable conclusion. Another example is Parkinson's disease (PD), in which two independent reports suggested that the risk of Parkinson's disease, at least in certain ethnic groups, was found to be associated with polymorphisms of ABCB1 (Furuno et al. 2002; Lee et al. 2004). However, a recent study by Kiyohard et al. (2013) found no significant correlation between the C3435T polymorphism and risk of onset of PD. These examples demonstrate the current state of ambiguity concerning the clinical impact of ABCB1 polymorphisms.

Meta-Analysis of ABCB1 Polymorphisms and Phenotypes

One of the major problems in clinical studies of ABCB1 polymorphisms has been poor sample size. Meta-analysis, which harnesses statistical methods to contrast and combine results from a pool of studies, is one way to address this problem. The lack of clarity concerning the association of ABCB1 polymorphisms and drug response/disease risk has led to meta-analysis in different clinical arenas, from cancer (Sheng et al. 2012) to epilepsy (Li et al. 2014). Onnie et al. (2006) published the first of this kind of report in 2006, showing that ABCB1 polymorphisms were associated with a small increase in the risk of developing ulcerative colitis and may influence disease behavior. In more recent years, meta-analysis has been used to determine ABCB1 association with drug resistance in epilepsy, cancer drug response, cancer risk, etc. (Table 2). These reports have established more clearly the association of ABCB1 and disease risk. In recent reports, the C3435T SNP was found to be associated with antiepileptic drug resistance (Li et al. 2014; Lv et al. 2014), and with better response to cyclosporin (Luo et al. 2012) and tacrolimus (Li et al. 2012) in transplant patients. Also, the C1236T SNP was found to be related to

Table 2 Meta-analysis of ABCB1 polymorphisms and disease risk or drug response

Reference	Subject	From	To	Studies	Samples	Major findings
Li et al. (2014)	Drug resistance in epilepsy	2003	2011	38	8716	C3435T associated with risk of antiepileptic drug resistance in Caucasians
Lv et al. (2014)	Drug resistance in epilepsy	2003	2011	23	8331	C3435T associated with drug resistance in epilepsy in Caucasians
Cheng et al. (2014)	Epilepsy	2005	2013	22	4269	3435TT lowers risk of resistance to antiepileptic drugs in Chinese populations
Dennis et al. (2014)	Opioid addiction and methadone metabolism	2005	2011	7	1052	No significant association with C3435T
Zu et al. (2014)	CML response to Imatinib	2008	2013	14	2997	CC1236TT increases risk of imatinib resistance in Asian CML patients
Zhang et al. (2015)	CML response to Imatinib	2008	2013	12	1826	CC1236TT increases risk of imatinib resistance in Asian CML patients, G2677T/AG and 3435T allele predict a worse response to imatinib in CML patients
Lee et al. (2014)	CsA dose after kidney transplant	2001	2014	11	1293	C3435TC requires higher CsA dose to achieve target therapeutic concentration
Li et al. (2012)	Tacrolimus dose after kidney transplant	2003	2011	13	1327	C3435TC requires higher tacrolimus dose to achieve target therapeutic concentration

(continued)

Table 2 (continued)

Reference	Subject	From	To	Studies	Samples	Major findings
Luo et al. (2012)	Clopidogrel-treated patients	2009	2011	6	10,153	C3435T associated with risk of short-term recurrent ischemic events
Wang et al. (2014)	Ischemic heart disease risk	2009	2013	7	12,816	C3435T increases risk of myocardial infarction and acute coronary syndrome
Wang et al. (2014)	Inflammatory bowel disease (IBD)	2003	2012	13	11,052	No significant association with C3435T
Megías-Vericat et al. (2015)	AML response to cytarabine plus anthracycline	2006	2012	7	1241	CC1236TT, GG2677T/AT, C3435T increase overall survival in Caucasians
Wu et al. (2014)	Gastric cancer risk	2007	2012	6	1220	No significant association with C3435T
Wu et al. (2014)	Peptic ulcer risk	2007	2012	6	1102	No significant association with C3435T
Doxani et al. (2013)	Acute myeloid leukemia (AML)	1992	2010	30	2094	No significant association with CC1236TT, GG2677T/AT, C3435T
Yao et al. (2014)	Breast cancer risk	2007	2012	9	12,571	3435TT vs TC + TC has a significant risk in Asian populations
Zhang et al. (2013)	Leukemia risk	2004	2012	11	4148	C3435T associated with risk of leukemia
He et al. (2013)	Colorectal cancer risk	2005	2011	10	6890	No significant association with CC1236TT, GG2677T/AT, C3435T

(continued)

Table 2 (continued)

Reference	Subject	From	To	Studies	Samples	Major findings
Zhao et al. (2013)	Colorectal cancer risk	2005	2013	13	11,339	No significant association with C3435T
Wang et al. (2012)	Cancer risk	2002	2010	34	6190	C3435T associated with cancer susceptibility, risk of breast and renal cancer
Sheng et al. (2012)	Cancer risk	2002	2010	39	22,767	C3435T associated with risk of blood, breast and renal cancer

risk in imatinib resistance in Asian CML patients (Zu et al. 2014). However, one recent meta-analysis indicated that ABCB1 SNPs could not actually predict the occurrence of IBD (Wang et al. 2014), response to therapy in Acute Myeloid Leukemia (AML) (Doxani et al. 2013) or colorectal cancer susceptibility (Zhao et al. 2013).

Although meta-analysis is a powerful approach, certain limitations of this approach should be taken into consideration, including data accuracy, the number of studies, the number of subjects included in the meta-analysis, as well as the different types of statistical analyses employed. Current experimental approaches and remedies can only partially solve some of these problems. To convincingly determine if an ABCB1 genotype might be associated with a particular phenotype such as disease risk or treatment response, pharmacogenomic research should be conducted with (1) a large number of high-quality samples, (2) genotype data conforming to quality assurance criteria, and (3) rigorous statistical analysis. For example, two large-scale, independent studies concluded that ABCB1 polymorphisms are not associated with ovarian cancer outcome (Johnatty et al. 2013; White et al. 2013).

In Vitro Analysis of Synonymous and Non-synonymous Polymorphisms of ABCB1

Introduction

In 1986, our laboratory published a report indicating that increased expression of MDR1 occurs during initial drug selection, without gene amplification (Shen et al. 1986). This important observation suggested that genetic mutations might play an important role in the development of drug resistance. It was also found that a glycine to valine substitution at position 185 (Gly185Val) in a colchicine-selected KB cell

line showed a significant increase in colchicine resistance compared with other drugs such as vinblastine and doxorubicin, suggesting that the ABCB1 sequence could be influenced by environmental factors and that its substrate specificity could change (Kioka et al. 1989; Choi et al. 1988). These early reports drove the search for functional ABCB1 gene mutations, which has continued until now.

In Vitro Assessments of the Effects of ABCB1 Single Nucleotide Polymorphisms

With improvements in gene sequencing techniques, polymorphic sites of ABCB1 were gradually discovered (Mickley et al. 1998; Kim et al. 2001; Cascorbi et al. 2001; Decleves et al. 2000). However, the impact of these SNPs on the expression and function of ABCB1 was not clear. In the past 15 years, laboratories in various part of the world have investigated the impact of SNPs of ABCB1 using different approaches, from cell lines with transient to stable expression, from yeast to mammalian cell lines, and from single SNPs to haplotypes.

The possible functional impact of these SNPs was investigated in two studies. A total of seven ABCB1 mutant plasmids (Asn21Asp, Phe103Leu, Ser400Asn, Ala893Ser, and Ala998Thr) (Kimchi-Sarfaty et al. 2002) and (C1236T and C3435T) (Kimchi-Sarfaty et al. 2007) were created. They were transfected into HeLa cells using a vaccinia-virus expression system. It was found that all five recombinant mutant proteins were expressed properly on the cell surface and that their function was comparable to that of wild-type ABCB1 (Kimchi-Sarfaty et al. 2002, 2007). None of these SNPs significantly altered ABCB1 function.

In another transient expression study, the function of seven ABCB1 SNPs (Asn21Asp, Ser400Asn, Asn669Cys, Ala893Ser, Ala893Thr, Ser1141Thr, and Val1251Ile) was tested to determine any differences from wild-type ABCB1. Plasmids carrying these mutations were transfected into HEK293T cells and assayed for expression and function of ABCB1. This study found that these SNPs did not affect protein expression. However, researchers in our own laboratory found that intracellular accumulation of calcein-AM and/or BODIPY-FL-paclitaxel was altered by the Ala893Ser, Ala893Thr, and Val1251Ile polymorphisms (Gow et al. 2008). Similar results were found by Kim et al. (2001), who discovered that the G2677T/A mutation decreased accumulation of digoxin in vitro.

In two reports, researchers examined the effect of the Ser400Asn polymorphism. Wild-type and variant ABCB1 were expressed in LLC-PK1 polarized cells and were found to be localized on the apical membrane with no differences in total ABCB1 expression. However, functional assays revealed reduced rhodamine 123 transport (Woodahl et al. 2004). In contrast, the transport of HIV protease inhibitors was increased (Woodahl et al. 2005). These data suggested that this mutation might behave differently in different cell lines and therefore showed variations in ABCB1 transport function.

ABCB1 SNPs were also studied in yeast cells. In a study by Jeong et al., yeast with the Met89Thr, Leu662Arg, Arg669Cys, Ala893Ser, Trp1108Arg, and Ser1141Thr mutations showed increased resistance to anthracyclines, actinomycin D and valinomycin (Jeong et al. 2007). These findings are not consistent with other reports. Although yeast expression systems offer several advantages over mammalian expression cell lines, it is known that yeast responds to xenobiotics very differently from human cells. Therefore, such studies may not help to explain human disease or treatment outcomes.

One additional study was carried out using membrane vesicle preparations from High Five insect cells expressing ABCB1 (Schaefer et al. 2006). Results showed that the minor allele at position 893 had reduced transport function. When the wild-type (893alanine) was mutated to serine, maximal transport rates for vincristine changed significantly. Also, inhibition by digoxin, didanosine, and fexofenadine was least efficient in the 893Ser-carrying transporter. These data revealed that serine and threonine have different effects on ABCB1 function at this amino acid position.

Assessments of ABCB1 Haplotypes In Vitro

The strong linkage disequilibrium of the common ABCB1 haplotype suggests that these polymorphisms might collectively have an important functional impact on the protein. In a seminal study, our group revealed that haplotype ABCB1 has a different protein conformation, causing altered protein function in a substrate-dependent manner (Kimchi-Sarfaty et al. 2007). Using a vaccinia-virus expression system, we found that wild-type and haplotype ABCB1 could be transiently expressed in HeLa cells. Recombinant wild-type and haplotype cells expressed comparable amounts of ABCB1 protein on the cell surface and exhibited similar transport function when using rhodamine as the substrate. A functional difference in the haplotype was shown when the cells were incubated with the P-gp inhibitor cyclosporin A. Haplotype ABCB1-expressing cells also displayed altered susceptibility to verapamil but not to rapamycin. These changes in protein function were caused by differences in protein conformation. This was demonstrated by (1) observed differences in immunolabeling profiles with the monoclonal anti-ABCB1 UIC2 antibody and (2) a limited-tryptic-digestion experiment, which suggested that membrane-bound haplotype P-gp was folded in a conformation less favorable for trypsin to access enzymatic cleavage sites relative to wild-type. This difference could be reversed by verapamil, a P-gp modulator. Our study strongly suggested that synonymous mutations in the ABCB1 haplotype produce a subtle but measurable change in the substrate-binding site conformation, and we offered a plausible explanation concerning how a synonymous mutation in the exon region could affect protein function. In 2008, Gow et al. (2008) also studied the same P-gp haplotype in 293T cells. Although they found no differences between the cells expressing the haplotype and the wild-type, the transport of BODIPY-FL-paclitaxel

function was measurably different between wild-type ABCB1 and the transporter containing four mutations (Asn21Asp/C1236T/Ala893Ser/Thr/C3435T).

The above studies were performed in transient expression cell lines that are known to have certain limitations, including instability of protein expression for long periods of time, off-target effects, and transfection reagent-related toxicity. In order to better mimic a physiologically relevant environment to study functional differences between human wild-type and haplotype ABCB1, we developed stable LLC-PK1 cell lines expressing wild-type and haplotype ABCB1. We reported that the wild-type and haplotype MDR1 proteins showed differential function in a substrate-dependent manner. We also found that the C3435T polymorphism influences protein stability. However, protein expression, protein targeting, cell growth rate, and ATPase activity do not seem to be influenced by the synonymous mutations.

Because it is possible to culture these cell lines as impermeable monolayers, we are able to conduct basolateral to apical drug transport assays to quantitatively measure effects of the P-gp haplotype. For example, using [^{3}H]-paclitaxel, we showed that wild-type P-gp-expressing cells have greater transport than haplotype-expressing cells. Control cells, expressing a non-functional mutant of P-gp, did not show significant basolateral to apical transport of the drug (Fig. 1).

While in vitro drug transport assays have clearly indicated that the ABCB1 haplotype changes protein folding and drug transport function in a substrate-specific manner, at this time, existing clinical studies do not consistently recapitulate these in vitro observations. The consensus of in vitro studies is that the C3435T polymorphism does not alter protein expression, although this is not confirmed by some reports (Hoffmeyer et al. 2000; Kimchi-Sarfaty et al. 2007; Gow et al. 2008; Fung et al. 2014). Several factors may contribute to these differences, including selection of the host cell, test methodology, and possible off-target effects related to cell manipulation. In vitro cell-based assays allow researchers to measure and compare the impact of polymorphisms in an unbiased way. This is important,

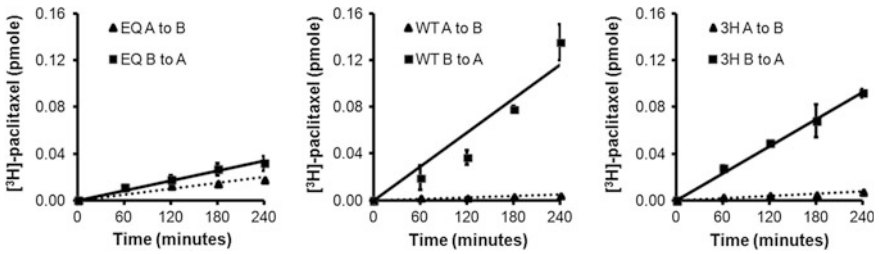

Fig. 1 Polymorphisms of P-gp influence cellular permeability of [^{3}H]-paclitaxel. Bidirectional transports of [^{3}H]NSC73306 across LLC-MDR-EQ (a functionally defective mutant (Sauna et al. 2007), LLC-MDR-WT, and LLC-MDR-3H cell monolayers were determined as a function of time. Apical to basolateral direction (*triangle*) and basolateral to apical direction (*square*)

as all of the human ABCB1 SNPs produce very subtle effects, and it should be remembered that these variants do not broadly inhibit transport function—instead, they commonly serve to modulate substrate specificity. Mammalian cell platforms expressing ABCB1 variants have been developed successfully, allowing some in vitro functional studies. However, most of these studies have focused on only a few SNPs (chiefly, C3435T). With the development of next generation sequencing technology, it is certain that more SNPs in ABCB1 will be identified. Given the broad impact of ABCB1 in normal and cancerous tissues, it is necessary to expand and extend research efforts in order to fully understand the role of its polymorphic forms.

Structural Insights into Polymorphisms of ABCB1

The Mouse abc1a Crystal Structure Resembles Human ABCB1

It is unfortunate that more than 40 years since the discovery of ABCB1, the exact molecular structure of human ABCB1 structure has not yet been determined. This has been attributed to the extreme conformational flexibility of this transporter. As an alternative, researchers have attempted to solve the structure of ABCB1 in other organisms. In 2009, Aller et al. reported the X-ray crystallography structure of *M. musculus* abcB1 at a resolution of 3.8–4.4 Å (pdb code: 3G5U) (Aller et al. 2009). At that time, there was a great interest in this structure since the mouse abcb1a amino acid sequence is 87 % identical to that of human ABCB1, so the murine structure was thought to closely resemble that of human ABCB1. The published apo-form structure showed an inward-facing inverted V-shaped conformation, which is different from the outward-facing model based on SAV1866, a bacterial homolog of ABCB1 (Dawson and Locher 2006). This shape creates a large internal cavity ($\sim$ 6000 Å), which is accessible to the plasma membrane inner leaflet and the cytoplasm, and is thought to bind molecules in different configurations. This hypothesis is supported by two additional abcB1 structures co-crystallized with the cyclic hexapeptide inhibitors cyclic-tris-(R)-valineselenazole (QZ59-RRR) and cyclic-tris-(S)-valineselenazole (QZ59-SSS), showing that the central cavity can bind to one ZQ59-RRR and two ZQ-59-SSS molecules. Together with the biochemical data available (reviewed in Ambudkar et al. 2006), this structure supports the polyspecificity of drug binding in the ABCB1 transporter. A follow-up report used mass spectroscopy to probe the intact ABCB1 small molecule-bound complex in detergent micelles. The results demonstrated that multiple states of conformation exist in equilibrium and they are readily interconverted by ligand binding (Marcoux et al. 2013).

Structural Impact of ABCB1 SNPs Using ABCB1 3D Structure

The impact on the globular structure of ABCB1 by SNPs is poorly understood. Most reviews merely outline the ABCB1 SNPs in a schematic diagram or two-dimensional cartoon of ABCB1 (Fung and Gottesman 2009; Meletiadis et al. 2006). This oversimplified approach does not indicate the true nature of the importance of ABCB1 SNPs. With the X-ray structure of mouse abcB1 now available, it is possible to mimic the human amino acid residues and map the residues harboring SNPs. In 2011, Wolf and colleagues published a review discussing the impact of ABCB1 SNPs using the mouse abcB1a 3D structure (Wolf et al. 2011). The authors generated a homology map with 62 SNPs in the coding region. Using this model, the authors indicated that the majority of mapped functionally associated SNPs reside in the two NBDs, and especially in NBD2. Most of the pharmacokinetic-related SNPs are found at the outer surface of the C-terminal NBD, with non-synonymous SNPs often found in less evolutionary-conserved residues. The conservation of amino acid residues comprising the haplotype is different in various species, with C3435T > G2677T/A > C1236T. For individual SNPs, Ser400Asn is a variable amino acid that resides in an evolutionary-conserved region, and the Gly185Val mutation resides in close proximity to the Ile144Tyr (10.9Å) and Asn183Ser (5Å) SNPs. The authors concluded that SNPs that alter protein function are found among poorly conserved amino acids and regions that allow higher tolerance toward changing amino acids. This might explain how the substrate specificity of ABCB1 can be changed by SNPs. However, this theory cannot explain why the C3435T polymorphism, which is the most commonly found SNP, is highly conserved among species.

Figure 2 shows a 3D structure of human P-glycoprotein based on the mouse 3D structure. The non-synonymous polymorphic sites (white spheres) and synonymous polymorphic sites (magenta spheres) are mapped. The SNPs found in this figure are listed in Table 3. Most of the synonymous SNPs are found in the NBDs, while non-synonymous SNPs are found in the NBDs and in the substrate-binding region. The alanine at 813 is found in TM9, located near the inner leaflet of the plasma membrane. Interestingly, the Thr173Pro SNP in TM3 is close to Ala813.

Higher Resolution Structures Needed

Since 2009, when the structure of mouse P-gp was published, the X-ray structures of other mammalian ABC transporters are still quite limited. ABCB1 is a highly flexible membrane protein and therefore poses a great challenge for biophysical studies using electron paramagnetic resonance (EPR), double electron–electron resonance (DEER), and fluorescence resonance energy transfer (FRET) techniques. Although the substrate-bound and inhibitor-bound forms of ABCB1 have been

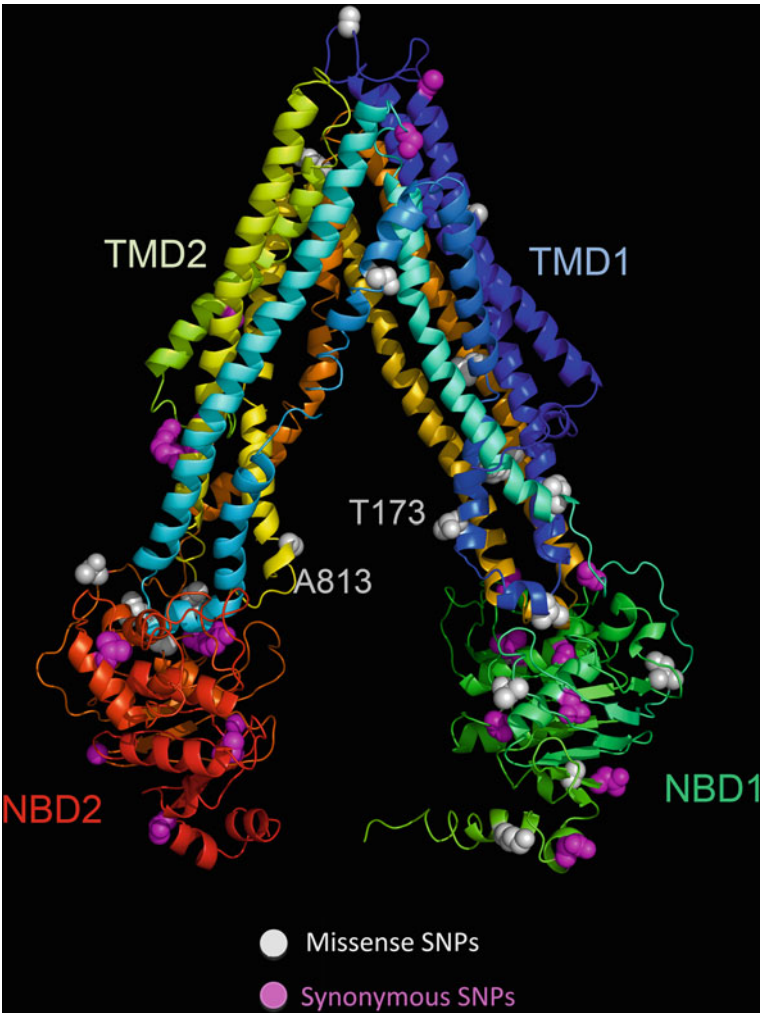

Fig. 2 3D structure of human ABCB1 showing locations of selected SNPs using the mouse ABCB1 inward-facing X-ray crystallography structure. The transmembrane domains and nucleotide-binding domains are shown in different colors. *Spheres* represent the non-synonymous polymorphisms (*white*) and synonymous polymorphisms (*pink*) listed in Table 2. The locations of amino acids at A813 and T173 are labeled

reported, the resolution of the ABCB1 X-ray structure still has much room for improvement. Only recently, a more refined structure of mouse abcB1 was published (Li et al. 2014). Without a high-resolution human ABCB1 structure, confirmatory site-directed mutagenesis studies of ABCB1 are still years away and the structural impact of ABCB1 polymorphisms is still very hypothetical.

Table 3 ABCB1 polymorphisms depicted in Fig. 2

Nucleotide position	Amino acid position	Rs number	Mutation	Nucleotide	Codon position	Amino acid change
4261	1256	rs149482536	Synonymous	G → A	3	T → T
4244	1251	rs28364274	Missense	G → A	1	V → I
4240	1249	rs2235051	Synonymous	C → G	3	G → G
4102	1203	rs200294906	Synonymous	G → T	3	T → T
3928	1145	rs1045642	Synonymous	T → C	3	I → I
3914	1141	rs2229107	Missense	T → A	1	S → T
3815	1108	rs35730308	Missense	T → C	1	W → R
3809	1106	rs148897157	Missense	G → A	1	V → I
3755	1088	rs57521326	Missense	G → A	1	D → N
3754	1087	rs200822574	Synonymous	C → T	3	Y → Y
3747	1085	rs201765972	Missense	G → A	2	R → Q
3682	1063	rs2707943	Synonymous	C → G	3	G → G
3644	1051	rs28401798	Missense	C → G	1	P → A
3562	1023	rs138566631	Synonymous	G → A	3	T → T
3561	1023	rs142183184	Missense	C → T	2	T → M
3411	973	rs149638669	Missense	A → C	2	D → A
3269	926	rs201316099	Missense	G → A	1	V → I
3170	893	rs386553610	Missense	T → G/A	1	S → A/T
2931	813	rs201744003	Missense	C → G	2	A → G
2891	800	rs41305517	Missense	G → A	1	D → N
2852	787	rs200903110	Synonymous	G → A	1	R → R
2647	718	rs199815160	Synonymous	C → T	3	A → A
2599	702	rs143151234	Synonymous	G → A	3	K → K
2499	669	rs146703713	Missense	G → T/A	2	R → L/H
2478	662	rs35657960	Missense	T → G	2	L → R
2442	650	rs200378616	Missense	C → G	2	A → G
2418	642	rs201352373	Missense	A → T	2	D → V
2364	624	rs141018820	Missense	A → G	2	K → R
2347	618	rs150277104	Synonymous	G → A	3	E → E
2288	599	rs2235036	Missense	G → A	1	A → T
2281	596	rs201194764	Synonymous	C → T	3	D → D
2167	558	rs56871767	Synonymous	G → A	3	T → T
2125	544	rs60247941	Synonymous	C → T	3	A → A
2110	539	rs35633772	Synonymous	C → T	3	I → I
2033	514	rs148455513	Missense	A → G	1	M → V
1942	483	rs201650388	Synonymous	G → A	3	T → T
1801	436	rs35068177	Synonymous	A → G	3	T → T
1729	412	rs386518005	Synonymous	T → C	3	G → G
1712	407	rs140214314	Missense	G → T	1	V → F
1692	400	rs2229109	Missense	G → T/A	2	S → I/N

(continued)

Table 3 (continued)

Nucleotide position	Amino acid position	Rs number	Mutation	Nucleotide	Codon position	Amino acid change
1656	388	rs149196148	Missense	T → G	2	I → S
1624	377	rs201349819	Synonymous	C → T	3	S → S
1598	369	rs199766539	Missense	A → G	1	I → V
1474	327	rs200490161	Synonymous	T → C	3	S → S
1157	222	rs138302009	Missense	A → G	1	S → G
1057	188	rs143782625	Missense	C → G	3	D → E
1047	185	rs1128501	Missense	G → T	2	G → V
1041	183	rs60419673	Missense	A → G	2	N → S
1029	179	rs201302394	Missense	T → C	2	V → A
1010	173	rs200753045	Missense	A → C	1	T → P
973	160	rs200823786	Missense	A → G	3	I → M
852	120	rs201352004	Missense	G → A	2	G → E
811	106	rs144036454	Synonymous	T → C	3	N → N
759	89	rs35810889	Missense	T → C	2	M → T
554	21	rs9282564	Missense	A → G	1	N → D
548	19	rs41304191	Missense	C → T/G	1	L → L/V
517	8	rs146259092	Missense	G → T	3	K → N

Effect of Synonymous Polymorphisms in ABCB1

Overview

Given the important functions this transporter performs within varied tissues and organ systems, the existence of clinically significant, disease, or treatment-modifying polymorphisms in ABCB1 is not surprising. However, the unforeseen ability of both non-synonymous (e.g., G2677T/A) *and* synonymous variants of ABCB1 (e.g., C1236T, C3435T) to modulate protein expression and transporter function has proven a pivotal discovery, engendering more broadly renewed investigative attention to an underappreciated class of genetic variants. Although encoding an identical primary amino acid sequence, evidence from molecular evolution unmistakably speaks to the importance of synonymous variants. Impressively, even at the laboratory timescale, directed evolution has demonstrated the significant impact these mutations can have on organismal fitness, where they may exert effects equivalent to those of their non-synonymous counterparts (Bailey et al. 2014). While their "molecular footprint" seen in the form of codon usage bias across genomic coding regions is well recognized and described (Plotkin and Kudla 2010), our understanding of the exact mechanisms by which

individual synonymous mutations or polymorphisms shape protein function, cell biology, and human disease has more slowly developed. Given the contribution of synonymous mutations in ABCB1 toward this understanding, it is worth highlighting the diverse ways by which synonymous nucleotide substitutions within open reading frames can impact gene expression and protein character.

The Cause of Change Lies in RNA

Anfinsen's principle states that protein structure is determined principally by its amino acid sequence (Anfinsen 1973). Yet, we know that the 3D structure and abundance of a given protein are nonetheless subject to the influence of synonymous mutations. Perhaps the most straightforward means by which these mutations exert their impact is through the influence of pre-mRNA splicing. While consensus splice sites occur at the end of introns in human genes, exonic splicing enhancers and silencers also contribute meaningfully to the recruitment of splicing machinery, as evidenced by the retarded rate of evolution and skewed codon usage at synonymous sites within ESEs (Parmley and Hurst 2007; Parmley et al. 2006) and the detailed instances of synonymous mutations underlying aberrant splicing and disease (Macaya et al. 2009; Nielsen et al. 2007). Similarly, synonymous mutations or polymorphisms falling within exonic transcription factor or micro-RNA binding sites can modify the binding of *trans*-acting regulatory elements, leading to dysregulated expression and the initiation of disease, with two such examples of synonymous mutations recently described to be associated with Crohn's disease and melanoma (Brest et al. 2011; Gartner et al. 2013). Without discounting these molecular mechanisms, synonymous polymorphisms in ABCB1 have mostly been credited to influence both protein expression and functionality by less indirect means.

Recent models have strongly implicated mRNA structure in determining ultimate protein expression (Kudla et al. 2009). While the complex three-dimensional structure of eukaryotic mRNA is dynamic and subject to a multitude of *cis*- and *trans*-elements, nucleotide base pairing forms a foundation for the thermodynamic stability or structural tendencies of an mRNA molecule. Altering these structural elements through differential base pairing allows synonymous polymorphisms and mutations to control not only the sheer robustness of translation (Nackley et al. 2006) but also the local kinetics of translation and co-translational folding (Bartoszewski et al. 2010). Paramount to the discussion of ABCB1, synonymous variants can also modulate the rate of translation by a second avenue: the non-uniform population of isoaccepting tRNAs. The choice among synonymous codons is non-trivial. Variation in tRNA abundance has been described at the species level and even among human tissues (Dittmar et al. 2006; Ikemura 1985).

The so-called "rare" or "frequent" synonymous codons for which there exists a limited or abundant supply of cellular tRNA may be selected for their ability to modulate the local speed of translation, which can be critical for the proper coordination of co-translational folding. Synonymous codon substitutions, especially those falling at critical locations along the open reading frame, can therefore alter the structural conformation and functionality of the encoded protein by shifting local folding minima. In this "translational stalling" theory, translation of amino acids from the mRNA by the ribosome complex is dependent on the time required for the aminoacyl-tRNA docking to the ribosome A-site. A nucleotide mutation that changes the codon sequence could alter this time. In fact, a recent study in yeast demonstrated that codon usage influences translational elongation rate (Presnyak et al. 2015). Thus, change in codon frequencies of ABCB1 in C1236T (from 0.33 to 0.18) and C3435T (0.52 to 0.35) might lead to changes in protein translation speed. Besides, nucleotide mutations could produce/eliminate mRNA secondary structures such as hairpins (Wen et al. 2008) or pseudoknots (Moran et al. 2008). Any changes in these mRNA structures could impact ribosome movement and therefore, ultimately change the general protein conformation. Although this theory could explain folding alterations in a local area, it cannot fully explain why a synonymous mutation could change folding in a distant area, such as in ABCB1. Nevertheless, such an idea has not been experimentally proven, and further exploration is warranted.

Conclusions

The field of ABCB1 pharmacogenetics has become a new frontier in drug transporter research. Exhaustive identification of functionally important SNPs in human ABCB1 might one day guide clinicians in making personalized decisions concerning drug treatment, disease prognostication, and risk stratification. In vitro studies clearly indicate that synonymous mutations in the coding region of ABCB1 influence multiple levels of biology, spanning RNA structure, transporter structure, and substrate specificity. Many of these polymorphisms are likely to differentially affect drug bioavailability within distinct populations. Unfortunately, data from clinical studies remains inconclusive at this time. Future efforts should therefore focus on large, standardized clinical observations and trials in order to accurately evaluate the pharmacogenomics of ABCB1. This approach might pave the way for the development of personalized therapies that harness our expanding capability to readily generate genotypic information.

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Interaction of ABC Transporters with Drugs

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Abstract ATP-binding cassette (ABC) proteins are causally related to the etiology of more than 25 human diseases. Selected members of the protein family play a role in drug disposition, drug resistance, and disease progression. The capability of ABC proteins to interact with small molecules has been well documented. An understanding of the mode of interaction of ABC proteins with drugs is an important aim for molecular and clinical pharmacology. The interaction of ABC proteins with drugs or drug candidates has been studied by structure-based and ligand-based approaches. In rare instances, a combination of both approaches has been attempted. Structure-based techniques rely on the availability of structural models of target ABC proteins at atomic resolution. Ligand-based studies infer information about drug-binding sites by complementarity between shape and properties of ligands on the one hand and binding sites on the other hand. These techniques are expected to lead to the identification of drug candidates for the treatment of those diseases, which are associated with ABC protein malfunction, but presently are not amenable to pharmacotherapy.

ABC Proteins in Human Physiology and Disease

Most of the human ABC proteins are transporters devoted to the uphill movement of cargo across biological membranes at the expense of ATP hydrolysis. However, other functions include ion conductance and ion channel regulation. Several ABC transporters, primarily ABCB1, ABCC1, and ABCG2, are involved in multispecific drug transport and therefore play a key role in drug disposition. Eighteen ABC proteins have been linked to more than 25 human disease etiologies (Linton et al.

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2011). Most of these diseases are rare, with the exception of cystic fibrosis, which represents the most frequent lethal inherited disorder in humans. Chapter 13 of this volume is dedicated to this disease. Despite considerable efforts of the scientific community, bringing together expertise in molecular, structural, and computational biology, as well as biophysics, the understanding of the functional biology of ABC exporters and their interaction with small molecules is still incomplete. Unresolved issues include the coupling of ATP binding/hydrolysis to cargo transport, nature, and location of substrate selectivity filters, interaction of cargo with the transmembrane domains (TMDs), nature and operation of the gates, and a detailed concept for alternating access (Jardetzky 1966).

The following lines of evidence identify ABC proteins as drug targets: First, multidrug resistance transporters bind and transport a wide variety of diverse chemical scaffolds. This makes them important players for pharmacokinetics of systemically administered drugs (Giacomini et al. 2010). Second, the bile salt export pump ABCB11 represents an off-target for drugs, including thiazolidinedione antidiabetics, protease inhibitors, endothelin antagonists, sulfonylureas, antibiotics, and kinase inhibitors (Dawson et al. 2012; Morgan et al. 2010). Third, the first drug targeting an ABC protein is as a potentiator for a variant of CFTR (ABCC7), which has been approved for use in patients recently (Hadida et al. 2014).

Publication of the first bacterial ABC exporter structure from *Staphylococcus aureus*, SAV1866, at 3.0 Å marked a major advance in the field (Dawson and Locher 2006). This multidrug transporter served as a paradigm for subsequently resolved structures, which, although differing in important details, show a common overall architecture. The most remarkable hallmark of these structures is a partial domain swap, where in contrast to the side-by-side orientation of ABC importers, each individual TMD forms contacts with both the ipsilateral and the contralateral nucleotide-binding domain (NBD).

The minimum functional unit of an ABC transporter consists of two TMDs, each of which contains six transmembrane helices, and two eponymous ATP binding cassettes, referred to as NBDs. In human ABC exporters at least one TMD and one NBD are contained in a single polypeptide chain. In addition, non-covalent interactions are formed between so-called coupling helices of intracellular loops (ICLs) of the TMDs and the membrane-oriented surfaces of the NBDs (Hollenstein et al. 2007). Hence, four ICLs of a functional transporter act as levers, which transmit conformational changes of the NBDs to the TMDs via their contact interface, the so-called transmission interface. The latter represents a weak point in the structure of ABC transporters, which is frequently targeted by mutations in patients suffering from diseases related to the malfunction of ABC transporters (Chiba et al. 2014; Nakagawa et al. 2011; Rudashevskaya et al. 2014).

Defining Drug/ABC Protein Interactions

Theoretical Considerations

All presently available ABC exporter structures show rotational (C_2) symmetry with respect to an axis that runs perpendicular to the membrane plane. In the apo state, this symmetry is ideal in homodimeric half transporters and retained to different extent in full transporters. The latter are considered to have arisen from half transporters by gene duplication (Gottesman and Pastan 1993). Rotational symmetry of homodimeric half transporters necessitates a dual binding mode of ligands, because each set of interacting residues in the functional dimer is represented twice. In other words, drug binding can occur randomly in one of the two binding modes, which are related to each other by 180° rotational symmetry (Fig. 1). Importantly, this basic concept is readily apparent from available crystal structures without the need to consider nature, chemical structure, or interaction principle of drug ligands.

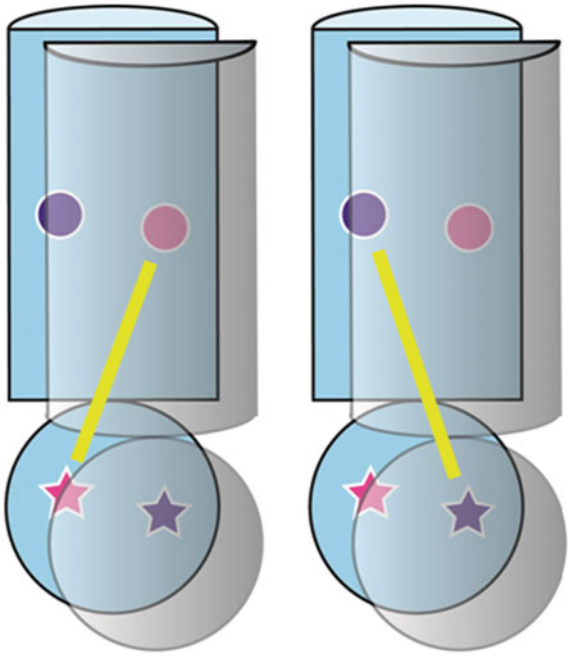

Fig. 1 Dual mode of substrate binding to the TMDs: The two halves of an ABC transporter are depicted in *gray* and *cyan*. The TMDs are shown as *half cylinders*, the NBDs as *hemispheres*. Substrates bind at the interface between the two TMDs in two symmetry-related modes (symbolized by *red* and *dark blue circles*). A putative dedication of these substrate-binding modes to composite nucleotide-binding sites (*star symbols*) is indicated by *yellow lines*. In which way these binding modes relate to the previously reported positively cooperative R- and H-sites reported (Shapiro and Ling 1997) has to be defined

Any randomly chosen binding competent ligand would select a set of interacting amino acid residues based on shape complementarity and physicochemical properties and bind in one of the two rotationally symmetric modes. Notably, this consideration does not pass judgment on the number of different binding sites, which may be provided for identical chemical entities, other than arguing the existence of a minimum of two rotationally related sites for each of them. The same consideration applies to secondary binding sites. Moreover, the dual binding mode concept does not intend to discuss, if binding modes are addressed simultaneously or sequentially. For half transporters such as LmrA, positive cooperativity of substrate binding has been reported (van Veen et al. 2000). This observation substantiates a dual binding mode.

In a next step, the situation in full transporters is considered. Here evolution shaped initially identical sites to become different, while retaining a variable degree of similarity. This makes the binding modes biochemically discriminable. Why evolution favored the emergence of full transporters remains unclear. Hypothetically, it might have served the expansion of the chemical space of transported cargo for multidrug transporters. This notion seems to be supported by data showing the preference of different chemical entities for one of the two binding modes in ABCB1 as discussed in detail below (Parveen et al. 2011).

While transporters are (pseudo)symmetric structures, they become completely asymmetric in action (Al-Shawi 2011). Marwan Al-Shawi pointed out that an alternating asymmetric power stroke would not be able to drive a symmetric alternating access mechanism without violating the principles of coupling. This incongruity, however, is resolved by the concept of a dual cargo-binding mode, which recapitulates the dual binding mode of ATP in the NBDs. Under the assumption that a dedication of either of the two nucleotide binding sites to one of these two binding modes exists, substrate binding in one mode would result in ATP occlusion and hydrolysis in one nucleotide-binding site (NBS), while hydrolysis would occur in the other, when substrate is bound in the alternative mode (Fig. 1).

Crystal Structures of ABC Exporters

The small number of resolved membrane protein structures owes to the fact that these proteins have to be removed from their native membrane environment prior to crystallization. This frequently precludes formation of high-quality crystals, which are required for atomic detail resolution. The dimensionality of the problem of resolving ABC protein structures is decreased to a certain extent, because membrane-spanning portions of the proteins are alpha helical. Accordingly, helices, which line the translocation path, may be assumed to require helical periodicity of polar and nonpolar residues. Indeed such a pattern can be seen for most membrane-spanning pore-exposed alpha helices. Arginine scanning has confirmed this periodicity for ABCB1 (Loo et al. 2009).

Once solubilized in detergent, ABC proteins have been shown to be highly dynamic structures. Crystallography thus often resorts to exploiting the ability of small molecules with specific binding properties to freeze conformations of membrane proteins. Accordingly, several structures of ABC transporters have been obtained in the presence of nucleotides. Alternatively, the apo state of the homolog of ABCB1 from the red alga *Cyanidioschizon merolae* was conformationally stabilized by use of a small peptide that tightly binds to the extracellular portion of the protein (Kodan et al. 2014).

As of April 2015, a total of 37 ABC exporter structures have been deposited in the protein data bank, which correspond to 9 unique proteins from 12 different species. Seventeen structures are of bacterial (Choudhury et al. 2014; Dawson and Locher 2006; Hohl et al. 2012; Lee et al. 2014; Ward et al. 2007), 20 of eukaryotic origin (Kodan et al. 2014; Li et al. 2014; Shintre et al. 2013; Srinivasan et al. 2014; Ward et al. 2013). As indicated, transporters are composed of two halves, which are either identical or similar. In the former case, proteins have to homodimerize to become functional. Heterodimerization is also observed, though at low frequency. Full transporters are thought to have arisen from half transporters by gene duplication. In the latter case, two TMDs and two nucleotide-binding domains are fused into one single polypeptide chain in the order TMD1-NBD1-TMD2-NBD2. Not surprisingly, the linker region connecting the two tandem TMD-NBD halves is the most diverse sequence stretch in full transporters. All human ABCA and ABCC subfamily members and four transporters of the ABCB subfamily (members ABCB1, ABCB4, ABCB5, and ABCB11) are full transporters. At present, X-ray structures of seven half transporters (Sav1866, MsbA, NaAtm1, Atm1, ABCB10, CmABCB1, and McjD) and two full transporters (mouse mdr1a and *Caenorhabditis elegans* P-glycoprotein) are available.

One structure is heterodimeric (TM287/288). A hallmark of the latter is that one of the two composite nucleotide-binding sites, which is formed at the NBD–NBD interface, is non-canonical. The catalytic glutamate of the Walker B motif of TM288 is replaced by an aspartate residue, rendering this NBS hydrolysis incompetent. Corresponding changes are found in the opposed signature (C-) sequence of TM287, which harbors mutations from a canonical LSGGQ sequence to LSTGE. With its degenerate nucleotide-binding site this transporter represents a paradigm for human ABC subfamily C members, the bile salt export pump (BSEP, ABCB11) (Childs et al. 1995; Gerloff et al. 1998), as well as the heterodimeric member of subfamily B TAP1/2 (ABCB2/B3) (Parcej and Tampe 2010).

About half of the available crystal structures have been resolved in the absence of nucleotide (apo state). The other half has been obtained in the presence of nucleotides or nucleotide analogs. Only mouse mdr1a has been co-crystallized in the presence of low molecular weight ligands (the cyclic peptide inhibitor isomers QZ59-RRR and QZ59-SSS) (Aller et al. 2009). Originally published structures have been revised (Li et al. 2014; Ward et al. 2013) making them compatible with biochemical evidence from arginine scanning experiments (Loo et al. 2009). Nucleotide-free structures show different degrees of separation of the NBDs (Ward et al. 2013). Even though distances might be more pronounced in crystal structures

obtained in the absence of membrane lipids, these snapshots of the transporters nevertheless define axes, along which motions of the transporters are possible. Accordingly, three directions of protein motion can be defined: (i) Association/disassociation of NBDs while TMDs are held together by loops at the extracytoplasmic face. These structures represent inward facing conformations. (ii) Separation of the membrane-spanning helical bundle into wing-like extensions at the extracytoplasmic face, as observed for the paradigmatic Sav1866 structures 2HYD and 2ONJ (Dawson and Locher 2006, 2007). Here helical bundles comprise helices 1 and 2 of one TMD and helices 3–6 of the other; these structures are thought to represent the outward facing conformation. (iii) A translational (shear) movement of NBDs relative to each other. Here some structures show Walker A motifs of both NBDs opposite each other in a nucleotide-free state, while the Walker A motif of one NBD faces the signature sequence of the other in nucleotide-bound structures. The former has been observed for nucleotide-free MsbA (Ward et al. 2007). Interestingly, a lower resolution electron cryomicroscopy structure for the multidrug transporter TmrAB from *Thermus thermophilus* also shows this positioning of the NBDs (Kim et al. 2015).

Binding of Substrates Occurs at the TMD Interface

Co-crystals of mouse mdr1a and the cyclic peptide inhibitors QZ59-RRR and QZ59-SSS show these compounds to be bound by contributions from both TMDs. The notion that substrates are bound at the interface between the two TMDs is also supported by biochemical experiments. Techniques include electron paramagnetic resonance (EPR) (van Wonderen et al. 2014; Wen et al. 2013), photolabeling (reviewed in Peer et al. 2005), mass spectrometry (Parveen et al. 2011; Pleban et al. 2005), cysteine (Loo and Clarke 1997, 1999, 2000) and arginine scanning, and cysteine cross-linking studies (Loo et al. 2004a, b, 2005; Loo and Clarke 1996, 2001, 2002, 2005). Several of these studies have provided supportive evidence for the notion that binding of substrates indeed occurs at the TMD/TMD interface.

Information on Substrate Interacting Amino Acid Residues from Site-Directed Mutagenesis Experiments

The largest body of experimental evidence to date has been compiled for the human multispecific drug efflux transporter ABCB1. Here structural visualization and projection of biochemical evidence in three-dimensional space relies on protein homology modeling (Chufan et al. 2013; Kerr et al. 2010).

In accordance with crystallographic data for mouse P-gp, site-directed mutagenesis experiments also provide evidence that the translocation path for solutes is

lined by residues from both TMDs. Table 1 summarizes residues of ABCB1, which have been identified to interact with substrates. This information has previously been compiled in two recent and comprehensive reviews by the groups of Suresh Ambudkar and Ian Kerr (Chufan et al. 2015; Wong et al. 2014) and in the publication of the *C. elegans* ABCB1 structure by the group of Jue Chen (Jin et al. 2012).

Pseudosymmetry of portions of the membrane-spanning regions in corresponding helices of the N- and C-terminal halves of human ABCB1 is shown in Fig. 2. Residues can be seen to be located at a maximum distance of two helical turns (10.8 Å), but often are in identical or adjacent position. Helical periodicity is apparent. Distances between residues that bind identical substrates are in part too far to be compatible with the notion of single binding modes for cargo molecules (L65-F728 23 Å; S222-G872 39 Å; I306-F942 29 Å as indicated by homology models). This evidence thus provides supportive evidence for a dual binding mode of substrates to ABCB1.

A combined photolabeling/mass spectrometry approach directly demonstrated dual pseudosymmetric binding of photoactive propafenone derivatives to ABCB1 (Parveen et al. 2011; Pleban et al. 2005). The parental compound propafenone is a class Ic antiarrhythmic compound, which has been shown to be a substrate and inhibitor of huABCB1. Photolabeling occurred at the TMD interface in regions of helix 5 (and adjacent regions in helix 8), as well as in a symmetry-related manner in helix 11. The two binding modes can be addressed individually in functional assays (Parveen et al. 2011) by inserting positively charged arginine selector residues in TM-helices 2 and 8. In these mutant transporters, only one of the two binding modes can be addressed by positively charged substrates such as rhodamine 123, because the other one is prevented by charge repulsion. This experimental system allowed demonstrating that binding not only of propafenones, but also of rhodamine 123, vinblastine, and verapamil occurs in two symmetry-related modes. The apparent affinity of these two binding modes differs up to 50-fold. Notably, this type of behavior indicates symmetry-related binding modes, but does not refer to binding sites, which likely are multiple in multispecific drug efflux transporters (Chufan et al. 2013). The issue of polyspecificity of drug transporters has been addressed by several recent reviews (Chufan et al. 2015; Gutmann et al. 2010; Sharom 2014; Wong et al. 2014).

Ligand-Based Techniques

In the absence of high-resolution structures of the protein, ligand-based structure–activity relationship studies (SAR) as well as quantitative SAR studies (QSAR) are the methods of choice for identifying molecular features driving ligand–transporter interaction. For most of these studies, again ABCB1 served as a model system. Ligand-based approaches rely on the assumption that pharmacophoric features of small molecules are imaged in properties of amino acid residues in the interacting

Table 1 Residues identified by site-directed mutagenesis to be involved in substrate binding of ABCB1 (P-glycoprotein)

N-term					C-term				
aa	Pos	H	Drug	Reference	aa	Pos	H	Drug	Reference
L	65	1	Ver, CsA, Vbl	Loo et al. (2006), Loo et al. (2007)	F	728	7	QCTVF	Chufan et al. (2013)
T	199	3	Vbl	Loo et al. (2007)	A	841	9	rhB	Loo and Clarke (2002a)
S	222	4	Ver, Col, Vbl, QCTVF	Loo and Clarke (2000), Loo and Clarke (2001), Chufan et al. (2013)	G	872	10	Ver, Col, Vbl	Loo and Clarke (2000)
I	306	5	CsA, Ver, Col, Vbl	Loo et al. (2007), Loo and Clarke (2000), Loo et al. (2003), Loo et al. (2004a)	F	942	11	Ver, Col, Vbl	Loo and Clarke (1999)
					T	945	11	Ver, Col, Vbl	Loo and Clarke (1999)
L	339	6	CsA, Ver, Col, Vbl	Ma et al. (1997), Loo and Clarke (2001), Loo and Clarke (1997)	L	975	12	rhB	Loo and Clarke (2002a)
I	340	6	rhB	Loo and Clarke (2002a)	V	981	12	rhB, QCTVF	Chufan et al. (2013), Loo and Clarke (2002a)
A	342	6	Ver, Col, Vbl	Loo and Clarke (2001), Loo and Clarke (1997)	V	982	12	rhB, QCTVF	Chufan et al. (2013), Loo and Clarke (2002a)
F	343	6	CsA, rhB, QCTVF, FM	Loo et al. (2007), Chufan et al. (2013), Rothnie et al. (2004)	6	984	12	Ver	Loo and Clarke (2001)
					A	985	12	Ver, Col, Vbl	Loo and Clarke (1997)

Abbreviations are as follows: *aa* amino acid; *pos* position in the protein; *H* helix. Substrates: *ver* verapamil; *vbl* vinblastine; *col* colchicine; *CsA* cyclosporine A; *rhB* rhodamine B; *FM* fluoresceine maleimide. QCTVF refers to the following five compounds: QZ59, cyclosporine A, tariquidar, valinomycin, fluorosulfonyl benzoyl adenosine

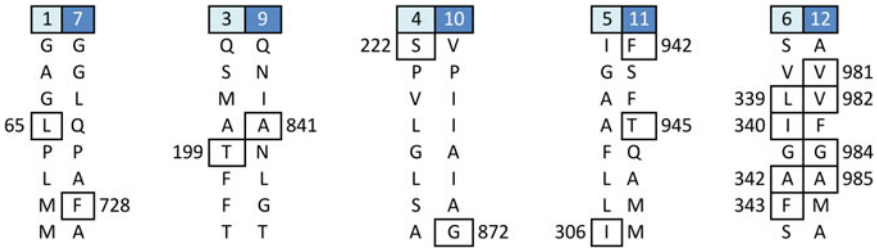

Fig. 2 Symmetry of residues contributing to substrate binding in ABCB1. Numbers in the *top row* refer to corresponding helices of the N-terminal half (*cyan*) and C-terminal half (*dark blue*). Alignments are top to bottom. *Boxed letters* and *numbers* refer to amino acids in single letter code and positions. Substrate binding residues are confined to approximately two turns within the membrane-spanning helices. Residue L975 is not shown

protein. Thus systematic structural modification of the ligand allows fathoming binding sites, which in this way can be explored and characterized. Undoubtedly, the fact that ABCB1 is multispecific cannot be deemed an asset. Nevertheless, conducting quantitative structure–activity studies proved feasible, as shown for multiple different chemical scaffolds (Bain et al. 1997; Honjo et al. 2006; Schultz et al. 2000).

2D- and 3D-QSAR Studies

QSAR is the most traditional method in ligand-based design. It was originally developed to obtain an understanding of the molecular features driving ligand–receptor interaction. In case of ABCB1, numerous successful SAR and QSAR studies have been published (Montanari and Ecker 2015). Interestingly, for each chemical series distinct molecular features for high and low inhibitory activity could be established. These features most often can be attributed to quite fundamental physicochemical parameters, such as lipophilicity, H-bonding, presence of aromatic ring systems, and charge state. 3D-QSAR studies such as CoMFA (comparative molecular field analysis), CoMSIA (comparative molecular similarity index analysis), as well as alignment-independent approaches using, e.g., GRIND or VOLSURF descriptors indicated convergence toward the same basic features as outlined above. Strikingly, there is still no general set of rules for designing in or out ABCB1 inhibitory potency. Furthermore, the concepts of ligand efficiency and lipophilic efficiency have to be applied in a different way than described for receptors (Jabeen et al. 2012).

Machine Learning Approaches

Availability of large data sets in the public domain made classification techniques for in silico profiling of compound libraries feasible. As for traditional QSAR, several different approaches have been pursued also in this field. They include rule-based algorithms, support vector machines (Klepsch et al. 2014), random forest classification (Poongavanam et al. 2012; Schwaha and Ecker 2011), as well as supervised and unsupervised neural networks (Thai and Ecker 2008). The great advantage of machine learning algorithms is their flexibility and their capability to deal with data of high complexity and high dimensionality. The major drawback is the lack of interpretability of most of the models, which hinders knowledge extraction. Furthermore, almost all methods need a proper definition of the applicability domain of the model, which is rarely considered by developers. Finally, it becomes increasingly evident that without proper standardization of transport assays, data extracted from the public domain have to be evaluated critically, requiring careful and extensive manual curation (Goldmann et al. 2014; Zdrazil et al. 2012).

Pharmacophore Modeling

For polyspecific proteins such as the hERG potassium channel or selected ABC transporters, pharmacophore modeling should be viewed as the method of choice for gaining insights into molecular features contributing to ligand–protein interaction. For creation of a pharmacophore model, individual chemical structures are reduced to interaction features (such as H-bond donor/acceptor, aromatic, hydrophobic, positive ionizable), and structurally diverse, active compounds are scanned for a common alignment of their features in 3D-space. Once a common pharmacophore is defined, it may serve for screening of large compound libraries to identify new chemical scaffolds. ABCB1 inhibitors served as the basis for development of several pharmacophore models, which show different, but partially overlapping features. The method has also successfully been applied for selectivity profiling of ABCB1 and ABCG2 ligands (Cramer et al. 2007). Most of the recently derived pharmacophore models show good capability for identifying ligands with new chemical scaffolds, thus proving their usefulness (Palmeira et al. 2011; Pan et al. 2013; Ritschel et al. 2014). However, due to high structural diversity of the ligands, also pharmacophore modeling so far did not lead to an improved molecular pharmaceutical understanding of the basis of polyspecificity.

Structure-Based Approaches

Availability of medium to high-resolution structures of several ABC transporters, which served as templates for the construction of homology models of human ABCB1, allowed structural information to be added to in silico models. For ABCB1, numerous docking studies of selected ligands into homology models were performed with the aim of identifying the molecular basis of ligand–transporter interaction (Chen et al. 2012; Montanari and Ecker 2015). Unfortunately, experimental validation of generated binding hypotheses is frequently missing. Recent advances focus on the use of structural information for virtual screening and classification, rather than proposing a concrete binding mode for a single ligand. Klepsch et al. used an experimental data-driven docking protocol developed for a series of propafenone analogs (Klepsch et al. 2011) to establish a structure-based classification model for a set of 1600 ABCB1 ligands. Although the overall accuracy of the model was worse than that obtained with random forest classification, it still showed significant enrichment (Klepsch et al. 2014). However, even if flexible docking protocols or induced fit algorithms are used, they only cover a very small part of the conformational space of the transporter. This prompts the move toward inclusion of molecular dynamics simulations in the analysis of ligand–transporter poses, as pursued by Ferreira (Ferreira et al. 2011). These studies convincingly demonstrate that structure-based modeling in the field of ABC transporters has become a valuable tool for a deeper understanding of the molecular features driving ligand–transporter interaction.

Areas of Pharmacological Application

The following areas of pharmacological development have in the past or are presently being pursued:

Multispecific drug efflux pumps and drug-resistant cancer

Multidrug resistance transporters have been found overexpressed in drug-resistant tumor cells. Their causal role in development of resistance toward antitumor agents, even when moderately overexpressed, has been confirmed in animal models and in human tumors (Rottenberg et al. 2007; Tamaki et al. 2011). Co-administration of standard anticancer drugs with inhibitors of these transporters has been advocated early on as a therapeutic concept for resensitization of resistant tumors. This concept has regrettably failed in clinical trials to date despite early encouraging reports due to initially unrecognized pharmacological interference of anticancer drugs and sensitizers at the level of the cytochrome P450 system. Causes for failure include life-threatening side effects due to myelosuppression as a consequence of ABCB1 inhibition and a lack of benefit in patient cohorts receiving co-treatment with P-gp inhibitors (Tamaki et al. 2011).

Compounds with the ability to selectively target P-gp expressing cells

In the NCI60 screen (Shoemaker 2006) an inverse correlation between expression of multidrug transporters and sensitivity of tumor cells to anticancer compounds is normally observed. Selected compounds however show a direct correlation between expression and sensitivity, i.e., the higher the expression of an ABC transporter; the more susceptible these cell lines are toward the action of those compounds. Though a reason for this phenomenon is presently elusive, specific targeting of cells expressing multidrug transporters may prove feasible (Szakacs et al. 2014).

Potentiators and correctors of ABC proteins

Often functional impairment, reduced, or absent trafficking of ABC transporters brings about a disease phenotype. In selected cases, small molecules with an ability to enhance function have been identified. An example of such a potentiator is Ivacaftor (Kalydeco), which has been brought to the market as a drug for treatment of cystic fibrosis (CF) in patients harboring the G551D mutation (Hadida et al. 2014). Though only a small number of CF patients benefits from this treatment, responses are impressive, encouraging the search for compounds with similar potentiating properties for other ABC transporter related diseases.

Another class of compounds shows corrector (pharmacological chaperone) activity for mutant ABC proteins that are unable to reach their correct subcellular localization due to impaired trafficking (see Chaps. 2 and 11). The root cause for these phenotypes is misfolding of the proteins due to point mutations. Here, mutant proteins are recognized as improperly folded and subsequently routed to the proteasome by a process called ER-associated degradation (ERAD). The latter has been identified as causally related to a number of disease phenotypes in at least 11 members of the ABC protein family (Chiba et al. 2014). Efforts to correct folding by using 4-phenylbutyrate as a chemical chaperone have been shown to be successful on an anecdotal basis for patients suffering from progressive familial intrahepatic cholestasis type II (Gonzales et al. 2012, 2015; Misawa et al. 2012). Ongoing clinical studies using pharmacological chaperones are presently confined to the CF transmembrane conductance regulator CFTR (see Chap. 11).

Designing in or out substrate properties during the drug development process

A notable example for exploiting substrate properties of P-glycoprotein is the third generation of antihistamines, which by attaining substrate properties for P-gp are prevented from entering the CNS. They are thus devoid of the sedative side effects associated with first and second generation drugs (Wang et al. 2001).

Concluding Remarks

Small molecules can alter the function of human ABC proteins and the pathophysiology of diseases associated with their malfunction. This makes ABC proteins druggable targets. Presently efforts are ongoing to translate preclinical concepts into

therapeutic strategies. Understanding the interaction of ABC transporters with small molecules might be viewed as one supportive effort in attaining this goal. Structural biology has provided higher resolution crystal structures, which have provided a basis for integration of available biochemical data, including those from site-directed mutagenesis, cysteine cross-linking, electron paramagnetic resonance, and photolabeling. Cornerstones for the interaction of drugs with model ABC proteins have been defined. P-glycoprotein undoubtedly is the most intensely studied member of the human ABC protein family to date. It might be expected to be representative for other ABC protein family members in certain aspects, but might fall short of providing guidance for others. Structure-based techniques have been complementing ligand-based studies. We expect that a combination of these techniques will eventually lead to an integrative understanding of cargo transport in a first member of the human ABC efflux transporter family at atomic detail. This is expected to have broad implications for the development of drugs with the potential to remedy medical conditions associated with the malfunction of human ABC proteins.

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Just How and Where Does P-glycoprotein Bind All Those Drugs?

Rituparna Mittra, Ellen M. Coyle and Richard Callaghan

Abstract P-glycoprotein (P-gp) was one of the first discovered, and most highly investigated, multidrug efflux pumps. P-gp was discovered in drug-resistant cancer cells and its ability to mediate adenosine triphosphate (ATP)-dependent efflux of drugs can confer resistance to cancer cells. The protein contains two sites for the binding and hydrolysis of ATP to power the active transport process. Drugs are known to bind within the transmembrane domain that comprises 12 membrane spanning α-helices. Biochemical, pharmacological and biophysical investigations continue to strive towards generating a molecular mechanism for drug transport. In addition, X-ray structures are available for the mouse and *Caenorhabditis elegans* isoforms at resolutions of 3–4 Å. However, one of the central issues related to the transport process remains elusive. A detailed understanding of how the protein is capable of binding its astonishing variety and number of compounds, remains unsolved. The *hydrophobic vacuum cleaner* and *drug flippase* models have been generated to describe this enigmatic property and some of their proposals remain intact. The majority of data supports the presence of a large binding domain that contains individual sites for drug interaction. These interaction sites are linked by an intricate allosteric network and binding to the sites is in close communication with the ATP hydrolytic machinery. This review provides a detailed account of our current understanding of how one membrane transporter is able to bind over 300 compounds.

Keywords P-glycoprotein · Multidrug resistance · Cancer chemotherapy · Membrane transport · Drug–protein interaction

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Introduction

Resistance to chemotherapy is either inherent to the cancer type or it is acquired in response to the chemotherapy regimen. The earliest reports of multidrug resistance used cancer cell lines selected for drug resistance by continuous exposure to anti-cancer drugs (Dano 1973). The resistant phenotype was characterised by a reduced accumulation of chemotherapeutic agents in cancer cells, which reduces the efficacy of cytotoxic anticancer drugs. The accumulation deficit was recognised as an ATP-dependent, protein mediated process and the culprit identified as the **P**ermeability **g**lyco**p**rotein, or P-glycoprotein (Ling and Thompson 1974).

P-glycoprotein (P-gp; also known as ABCB1 or mdr1) is a polyspecific membrane transporter belonging to the **A**TP-**B**inding **C**assette superfamily (Higgins 1992; Riordan and Ling 1985). It functions by actively effluxing compounds from within the cytosol and lipid bilayer to the external environment. Of the 48 known human ABC transporters, P-gp is arguably the most extensively researched as the expression of this protein in cancer cells plays a significant role in conferring multidrug resistance to cytotoxic anticancer drugs. P-gp has a molecular weight of 140 kD and is comprised of 1280 amino acids that are encoded as a single polypeptide (Chen et al. 1986; Ling et al. 1983). The protein is comprised of two homologous halves each containing a transmembrane domain (TMD) and a cytosolic nucleotide-binding domain (NBD). The former provides the conduit for drug transport and the latter generates energy from ATP hydrolysis for the process. Each transmembrane domain consists of six membrane spanning helices that display domain swapping and facilitate energetic coupling between nucleotide binding, substrate binding and nucleotide hydrolysis, by virtue of their direct contact to the catalytic sites, the NBDs.

Despite the extensive amount of information available on P-gp, there is a limited mechanistic understanding of its function. Of particular importance is how the protein can mediate the transport of such a large array of chemically and functionally unrelated drugs. P-gp appears to violate many of the "rules" of specificity that govern receptor, enzyme and transporter biology. It is by no means the only promiscuous member of the ABC superfamily of proteins and multidrug efflux systems exist throughout the biological world. The focus of this chapter is to outline the many different strategies that have been employed, in the 40 years since the discovery of P-gp, to address the key concept of how one protein is able to interact with such an extraordinary number of compounds.

Using Site-Directed Mutagenesis to Locate Drug-Binding Sites

The explosion of molecular biology techniques from the late 1980s was embraced in the search to define functionally important regions of P-gp; in particular, the drug-binding site. P-gp was mutated at specific amino-acid residues and the

functional consequence measured in order to attribute a specific role in the transport process. P-gp function may be measured at multiple levels; for example, cell cytotoxicity assays reveal the effects on the overall resistant phenotype. Cellular accumulation of radiolabelled or fluorescent drugs, provided an insight into the transport process. The effects of mutations on drug-stimulated ATPase activity and photo-affinity labelling techniques both demonstrate a direct interaction of drugs with P-gp. Finally, and perhaps most relevantly, the binding of drug to mutated protein can be measured with equilibrium assays to provide the true affinity constant (K_D) for the interaction.

Alanine Mutagenesis: An Early Approach

A theoretical study suggested that hydrophobic drug substrates of P-gp were likely to intercalate between phenylalanine (Phe) residues in the transmembrane (TM) helices (Pawagi et al. 1994), and in an early mutagenesis approach, the 31 intrinsic Phe residues were targeted. A total of 31 mutant versions of P-gp were generated, each containing a single Phe mutation to alanine and the proteins expressed in mouse NIH-3T3 cells (Loo and Clarke 1993). The mutant P-gp isoforms were analysed to assess whether the Phe → Ala mutation affected their ability to confer resistance to drugs (i.e. phenotypic assay) including vinblastine, actinomycin D, colchicine and adriamycin. A significantly different drug resistance profile was observed in only three of the mutants, while the other 28 resulted in protein products which were functionally and structurally indistinguishable from wild-type P-gp (Loo and Clarke 1993). The F777A (TM8) mutant exhibited a 2-fold decrease in the relative resistance to all four drugs and its effect was attributed to perturbed biosynthesis of P-gp due to lower level expression of this isoform. Mutant F335A (TM6) retained the ability to confer resistance to colchicine and adriamycin but conferred little resistance to actinomycin D or vinblastine. In contrast, mutant F978A (TM12) displayed little to no resistance to adriamycin or colchicine but was able to confer resistance to vinblastine or actinomycin D. Overall this investigation suggested that drugs may interact at distinct sites on the protein and that both halves of the polypeptide were involved in drug binding.

A number of other similar investigations were reported at this time, but with 1280 amino-acid residues, the possible permutations of amino-acid substitutions are daunting to say the least. A more systematic and directed mutagenesis approach was clearly required.

Cysteine Mutagenesis: A Widely Used Approach

The cysteine-scanning mutagenesis approach provides a systematic and flexible approach to elucidating the biochemical pharmacology of P-gp. The key requirement

to adopt this approach is that mutation of the intrinsic cysteine residues does not significantly perturb the function of P-gp. The seven intrinsic cysteine residues (at positions 137, 431, 717, 956, 1074, 1125 and 1227) were each mutated to alanine and generated a cysteine-less (cys-less) P-gp that was functioned identically to wild-type protein (Loo and Clarke 1995a). Another team substituted the intrinsic cysteines to serine and produced a fully functional cys-less P-gp isoform (Taylor et al. 2001).

The use of a cysteine-mutagenesis strategy provides twofold benefit (Frillingos et al. 1998). First, the effects of replacing a residue with cysteine in a specific region of the protein may perturb the overall transport capability of P-gp. This will provide insight into the "native" function of that specific residue and establish the local region for further mutagenesis. Alternatively, the mutation of a "native" residue to cysteine does not cause significant perturbation of function. This will enable the investigator to utilise the chemistry of cysteine; in particular, the ability to covalently modify the sulphydryl moiety under physiological conditions (e.g. pH 7.4, 37 °C). There are numerous sulphydryl-reactive compounds (e.g. maleimide or methanethiosulphonate (MTS) containing) with fluorescent, radiolabelled or spin-labelled probes attached. Covalent attachment of more bulky compounds may provide further local perturbation of function and the use of spectroscopic probes will enable biophysical investigation of local environment or structural changes in the protein. The focus of the subsequent four sections is the identification of residues within several transmembrane segments that may be involved in binding of drugs by P-gp.

Dibromobimane and Protection from Inhibition

P-gp contains a central cavity (see Sect. "Can Structural Information Locate Drug Binding Sites?") that is presumed to form the drug-binding domain and undergoes alternating access across the membrane during the transport process. The cysteine-scanning mutagenesis approach was used to identify residues lining the cavity that contribute to drug binding. In particular, the thiol-reactive compound, dibromobimane (dBBn), was reacted with a series of P-gp isoforms containing cysteine insertion into positions within TM6 and TM12 (Fig. 1) (Loo and Clarke 1997, 2000, 2002). Treatment of the mutant protein isoforms with dBBn impaired the drug-stimulated ATPase activity of P-gp, although this perturbation may be due to alteration of any of the multiple steps in the process (e.g. conformational change or drug binding). Consequently, the single-cysteine isoforms were pre-incubated with one of the substrates/modulators of P-gp prior to the addition of dBBn. If the drug pre-treatment prevented dBBn-induced inhibition of ATP hydrolysis (i.e. protection), then it may be inferred that the introduced cysteine residue is situated proximal to the binding site. Using the substrates vinblastine and colchicine, and the modulator verapamil, the authors concluded that residues mutated in TM6 (L339C and A342C) and in TM12 (L975C, V982C and A985C) were involved in the interaction of substrates with P-gp (Loo and Clarke 1997).

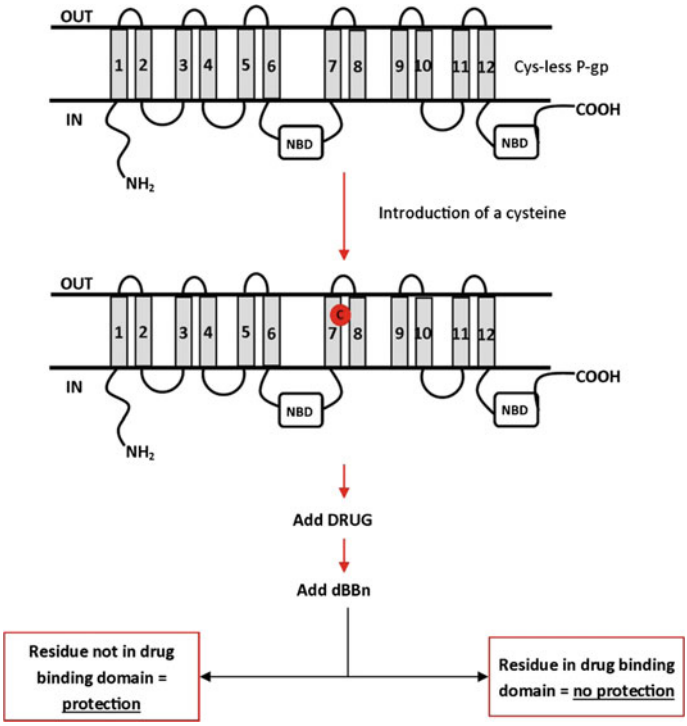

Fig. 1 Construction of Cys-less P-glycoprotein and cysteine-directed mutagenesis studies with dibromobimane (dBBn). Wild-type P-gp contains seven endogenous cysteine residues which were mutated to alanine resulting in a Cys-less P-gp construct. Residues in a number of transmembrane helices were mutated to cysteine and purified using nickel affinity chromatography by virtue of a C-terminal polyhistidine tag. Inhibition of verapamil-stimulated ATPase activity in the presence of the thiol-reactive cross-linking agent dBBn was measured to identify various residues important in drug binding

These initial studies prompted larger investigations to identify potential residues within TM segments that interact with substrates of P-gp and thereby refine the topography and the environment of the drug-binding pocket. A study published in 2000 analysed 189 cysteine mutations scattered around the TM segments of P-gp predicted to line the central cavity (i.e. TMs1–5 and 7–10) (Loo and Clarke 2000). The mutant isoforms were analysed for inhibition of drug-stimulated ATP hydrolysis by dBBn. Seven mutants, Y118C and V125C (TM2), S22C (TM4), I306C (TM5), S766C (TM9), and I868C and G872C (TM10), displayed considerable inhibition of verapamil-stimulated ATPase activity upon treatment with dBBn. However, protection from inhibition by dBBn following pre-treatment with vinblastine, colchicine or verapamil was only seen for mutants S222C (TM4), I306C (TM5), I868 (TM10) and G872C (TM10). These results and related investigations (Loo and Clarke 1997, 1999, 2000, 2002) conclude that transmembrane helices 4, 5, 6, 10, 11 and 12 are implicated in drug binding by P-gp.

Methanethiosulphonate Analogues and Protection Assays

Concurrent to the investigations with dBBn, an alternative strategy to elucidate residues within the binding site adopted a thiol-reactive derivative of verapamil (MTS-verapamil). The premise was to use a compound more similar to a substrate to achieve covalent modification and ensure specificity for the drug-binding domain. The methanethiosulphonate group was attached by an ethyl linker arm to the methylamine group in verapamil and the ATPase activity was measured in cys-less P-gp. MTS-verapamil produced a similar degree of ATPase stimulation, with equivalent potency, thereby retaining the ability to interact with P-gp (Loo and Clarke 2001).

MTS-verapamil was used initially to generate a covalent modification within the drug-binding domain and the effects of 252 single-cysteine mutations on the labelling were investigated (Loo and Clarke 2001). The covalent attachment of MTS-verapamil to P-gp caused a reduction in the ATPase activity of several mutant isoforms. Specifically, the reduction in ATPase activity was found in only 15 of the 252 mutants: Y118C (TM2), V125C (TM2), S222C (TM4), L339C (TM6), A342C (TM6), A729C (TM7), A841C (TM9), N842C (TM9), I868C (TM10), A871C (TM10), F942C (TM11), T945C (TM11), V982C (TM12), G984C (TM12), and A985C (TM12). It was argued that the use of a substrate with an attached MTS moiety was more likely to identify residues specifically within the binding domain. However, the possibility of the MTS-cysteine affinity driving the labelling cannot be unequivocally excluded. Why should attachment of MTS-verapamil cause a reduction in ATPase activity? This is presumably related to the need for dissociation of drug from the TMD to enable completion of the ATP hydrolytic process.

In order to further support the specificity of this approach, the authors also used a "verapamil protection" strategy to differentiate cysteines that are proximal to drug-binding sites from those that are not (Loo and Clarke 2002; Loo et al. 2003a, 2006a, b). This strategy was possible since both verapamil and its thiol-reactive analogue have similar K_m values (substrate affinity) for cys-less P-gp. Therefore, incubation with verapamil prior to addition of MTS-verapamil should protect the mutant from inactivation by MTS-verapamil if it is proximal to the binding site in question. Significant protection from inhibition by MTS-verapamil was found in four mutants (compared to 15 above); namely, S222C (TM4), L339C (TM6), A342C (TM6) and G984C (TM12). A lesser degree of protection was observed in mutants I868C (TM10), F942C (TM11) and T945C (TM11), which may indicate partial overlap of binding sites or a more peripheral location of the residues to the interaction site of verapamil. Overall, the results using MTS-verapamil have refined the number of helices putatively involved in the binding domain, but have strengthened the evidence that TMs 4, 6, 10, 11 and 12 contribute to the drug-binding pocket.

Verapamil is a well-established inhibitor of cytotoxic drug transport by P-gp and its administration with anticancer drugs is able to overcome the drug-resistant phenotype in vitro. It has been purported to be a transported substrate of P-gp, although the data is not conclusive to support this. Consequently, verapamil should be considered a "modulator" of P-gp function. In contrast, it is clear that several

rhodamine derivatives are direct substrates for P-gp mediated transport (Shapiro and Ling 1997b). Moreover, it may transpire that substrates and allosteric modulators bind at distinct sites on P-gp. Therefore an MTS derivative of rhodamine was also used to identify the drug-binding domain of P-gp. In a similar approach to that outlined in the preceding paragraph, 252 single-cysteine mutants were generated, reacted with MTS-rhodamine and their ATPase activities measured (Loo and Clarke 2002). Of these mutations only 28, which were located in TMs 2–12, were markedly inhibited following covalent attachment of MTS-rhodamine. Subsequently, pre-treatment with the parent compound rhodamine significantly protected the activities of five mutants, I340C (TM6), A841C (TM9), L975C (TM12), V981C (TM12) and V982C (TM12), implicating TMs 6, 9 and 12 in the binding of rhodamine dyes.

The "Herculean" efforts involving site-directed mutagenesis of the TM domains of P-gp have significantly narrowed the search for the location of drug-binding sites on P-gp. A number of helices and their constitutive residues, have been implicated using distinct investigative strategies. None of the studies identified precise locations for drug binding, but this is hardly surprising given the promiscuity of P-gp and the large number of helices involved in lining the binding domain or cavity. However, these biochemical investigations will be vital in conjunction with structural data. There remains a clear need for further, and multiple, approaches to locate and define drug binding on this transporter.

A Proposed "Substrate-Induced Fit" Model

The data described in the previous sections clearly demonstrate that multiple helices, presumably those lining the central cavity, contribute to drug binding or the formation of a binding domain. In addition, data obtained with dBBn and MTS-cross-linking substrates have revealed a number of common residues from these helices that interact with distinct P-gp substrates. Conversely, the data also indicated that distinct classes of drugs interact with different residues. This point was also supported from a series of investigations, also using a cysteine-mutagenesis strategy, that focussed specifically on the involvement of TM helices 6 and 12 (Rothnie et al. 2004; Storm et al. 2008; Crowley et al. 2009, 2010a). In summary, these investigations potentially generate a hypothesis that drugs bind at "*common, but different, sites*" on the protein, or that binding sites may have some overlap of residues.

Can this rather confusing observation be reconciled to a unifying description of drug binding to P-gp? Such a model does exist and has been termed the "*substrate-induced fit*" hypothesis (Loo et al. 2003c). According to this hypothesis, a common binding site exists for drugs in the central cavity of P-gp. Following interaction at the common residues, a combination of residues from different transmembrane helices is moulded by the substrate to form its specific drug binding site.

Such a model would require that the cavity and its lining helices are mobile and a disulphide cross-linking study suggests helical mobility is present in P-gp and that there is a great deal of evidence to support this. For example, electron microscopy (EM)-based structural studies (Rosenberg et al. 2001, 2003, 2004) and biophysical approaches with $^{2}H/^{1}H$ exchange kinetics (Sonveaux et al. 1996, 1999) revealed that drugs and nucleotides generate considerable rearrangement of the TMD structure. It is the nature of contributing residues from each TM helix that determines the affinity for the substrate (Loo and Clarke 2002). Another investigation using cysteine mutagenesis to explore the nature of cross-linking between helices in the presence and absence of drugs provides further support (Loo et al. 2003c). P-gp isoforms were constructed, each with a pair of cysteine residues introduced at distinct helices. Cross-linking was induced by addition of the oxidant copper phenanthroline in the absence or presence of the drug, whereby the two conditions generated markedly different cross-linking patterns. For example, in the presence of colchicine and demecolcine, cross-linking was observed between residues P350C (TM6)/V991C (TM12), whereas cyclosporin A promoted cross-linking between P350C (TM6)/G939 (TM11) only. The addition of progesterone produced a number of cross-links; namely, P350C (TM6)/A935C (TM11), P350C (TM6)/G939C (TM11), and between P350C (TM6)/V991C (TM12). In contrast, other modulators and substrates such as verapamil and vinblastine did not promote cross-linking for any of the mutant pairs investigated. This study showed direct evidence of re-packing of TM segments to form an 'induced' drug-binding site upon addition of particular substrates, to help explain how the protein accommodates such a vast range of compounds.

Evidence of Simultaneous Binding

Evidence presented thus far has suggested that P-gp has a central cavity that mediates substrate and modulator binding. The binding domain comprises several TM helices and has a malleable structure to accommodate the interaction with drugs of distinct chemical composition. Can this binding domain interact with more than one drug simultaneously?

To address this issue several mutant isoforms of P-gp were investigated for their interaction with the thiol-reactive substrate tris-(2-maleimidoethyl)amine (TMEA), which will cross-link to any cysteine close to its binding site (Loo et al. 2003b). If a second drug was to bind at the same time at an overlapping site (e.g. competitive binding), then it would inhibit binding and cross-linking of TMEA to the protein. However, if the second substrate was to bind at a distant site (i.e. allosteric), then the cross-linking caused by TMEA would be unaffected, or possibly enhanced. Using verapamil as the second substrate, it was shown that cross-linking could be induced between TM6 (F343C) and TM12 (V982C). This is in contrast to the absence of any cross-linking in the presence of TMEA alone and evidence of inter-site allosteric communication. This cross-linking study using a mutagenesis

approach therefore provided evidence in support of simultaneous binding of two substrates, each occupying a distinct region within the binding pocket (Loo et al. 2003b). However, this is not the only report of simultaneous binding of substrates to P-gp. Lugo and Sharom demonstrated that two fluorescent substrates (Rhodamine 123 and LDS-751) bound simultaneously to P-gp since they exhibited a spectral interaction upon binding to the protein (Lugo and Sharom 2005). In addition, classical receptor pharmacology studies demonstrated that the binding of one drug can accelerate the dissociation of another (Ferry et al. 1992; Martin et al. 1997). Altered dissociation rate constants can only occur with an allosteric interaction and therefore further proof of simultaneous drug interaction at the binding domain.

Photo-Affinity Labelling of Drug-Binding Sites

As described above, a vast amount of data has been acquired from the systematic insertion of cysteine mutants, followed by reaction with thiol-reactive substrates and cross-linking agents to characterise the large binding domain of P-gp. The data also identified the TM helices involved in mediating binding, provided an insight into some of the residues involved, and revealed the existence of multiple sites. However, the data on their own have not provided precise information on the sites of drug interaction. At the same time, a number of investigations used photo-activated probes and drugs in an attempt to find the drug-binding site(s) on P-gp.

The Anatomy of a Photo-Affinity Probe

The use of photo-affinity labelling to identify specific regions of a protein obviously requires a photo-active probe that binds to the target region. Following incubation of the photo-active probe with protein and binding equilibrium has been reached; the ternary complex is irradiated with light. Post-irradiation, the photo-active probe (bound at its target site) is converted to a short-lived, highly reactive nitrene intermediate that covalently attaches to the polypeptide chain. Due to a short half-life, these probes should efficiently label only specific sites that are in close proximity. The photo-affinity probes must be of high affinity to the target site, stable in an aqueous environment and display high specific radioactivity. Moreover, the photo-active compounds need to retain the activity or binding characteristics of the parent compound (Safa 1999). Once the photo-cross-linking has been achieved, there are a number of approaches to ascertain the site of labelling. However, great interpretive care must be exercised with this approach since the photo-active moiety may lie on a flexible region of the molecule and potentially label regions that lie external to the absolute binding site (Glossmann et al. 1987).

The first applications using photo-affinity labelling were to establish whether drug substrates and inhibitors bound to P-gp and to ascertain the potencies for drug binding to the protein (Greenberger 1993; Bruggemann et al. 1989; Hafkemeyer et al. 1998; Isenberg et al. 2001; Safa 1988; Zhang et al. 1995). Amongst the first photo-affinity probes to be used with P-gp were analogues of the well-characterised substrate vinblastine; *N*-(p-azido[3-^{125}I]salicyl)-*N*′-aminoethylvindesine ([^{125}I]NASV) and *N*-(p-azido[3,5-^{3}H]benzoyl)-*N*′-(beta-amino-ethyl)vindesine ([^{3}H]NABV) (Beck et al. 1988; Naito et al. 1989). The photo-affinity analogues of vinblastine were incubated with purified plasma membranes, mixed membrane vesicles or intact cells, and then irradiated for activation. Photo-affinity labelled protein samples were then analysed using SDS–PAGE followed by autoradiography to show that only resistant cell lines (overexpressing P-gp) were labelled with [^{125}I]NASV. It was demonstrated that in the presence of 100 μM vinblastine, [^{125}I]NASV photo-labelling was abrogated. This was the first demonstration that anticancer drugs bound directly to P-gp. In contrast to vinblastine, the anthracycline doxorubicin only produced partial inhibition of [^{125}I]NASV labelling, whereas colchicine and methotrexate (a drug to which MDR cells are sensitive) did not show any blocking of photo-labelling. This was interpreted to suggest that colchicine binds at a separate site to the vinblastine analogue. Alternatively, it merely demonstrates that colchicine has a lower affinity for the vinblastine-binding site and a rank order of potency was defined as vinblastine > vincristine > doxorubicin > actinomycin D > colchicine (Safa 1988; Safa et al. 1989).

Photo-Labelling and Protease or Chemical Cleavage Approaches to Locating the Drug-Binding Site of P-gp

An early photo-affinity labelling study (Bruggemann et al. 1989) attempted to identify the regions of P-gp that were labelled by [^{3}H]-azidopine. Azidopine is a photo-active dihydropyridine analogue that was initially developed to label L-type calcium channels (Ferry et al. 1985). The compound also inhibits the active transport of vinblastine in P-gp expressed in plasma membrane vesicles from the drug-resistant human carcinoma cell line, KB-V1 (Bruggemann et al. 1989). P-gp was photo-labelled with [^{3}H]-azidopine, the protein was then digested with trypsin and V8 protease to enable the identification of the labelled fragments using specific antibodies. P-gp was digested and two major fragments were detected by immunoblotting. A 38-kDa fragment containing one-third of the labelled [^{3}H]-azidopine was derived from the amino half, and a 30-kDa fragment containing two-thirds of the [^{3}H]-azidopine, from the carboxyl half (Bruggemann et al. 1989). Immunoprecipitation and endoglycosidase treatments were used to define that the 38-kDa tryptic fragment contained TM helices 1–6 and that the 30-kDa V8 fragment contained TM helices 7–12. Since two different regions of P-gp were labelled by [^{3}H]-azidopine, it is possible that the protein contains two binding sites for the

compound, one in the carboxyl half and the other in the amino half. The alternative interpretation was that P-gp contains one binding site that is formed by the two homologous halves of the protein and that these two regions come together to create a single binding pocket for [^{3}H]-azidopine.

A subsequent study used both [^{3}H]-azidopine and [^{125}I]-iodoaryl azidoprazosin ([^{125}I]-IAAP) ligands and a similar protein digestion protocol (Greenberger et al. 1990). Both photo-affinity probes bound to highly related, if not identical, domain on the protein despite the chemical dissimilarity of the compounds. One explanation was that although both molecules were structurally diverse, they both contained the reactive arylazido group and thereby labelled the same protein region. Alternatively, P-gp could accommodate structurally diverse molecules in its binding site. In a follow-up study, the 40-kDa photo-affinity labelled domain in the C-terminal half of P-gp (predicted to reside within TM11) was shown to contain a major 4-kDa photo-labelling site. Its identity was deduced by antibody-based precipitation and consisted of approximately 29 residues, located between residues 979 and 1048 (Greenberger 1993). Further analysis of protein fragments using protease and chemical cleavage approaches had begun to identify numerous regions of drug attachment. For example, a 6-kDa trypsin fragment (found using antibody 1) in a predicted location within TM4 and up to (but not including) TM6, and a minor 40-kDa photo-labelled fragment between TM7 and TM8, were identified. In addition, a 25-kDa cyanogen bromide fragment was generated, containing an azidopine-labelled domain spanning the region from TM4 to the Walker A motif, (Bruggemann et al. 1992). Therefore, this molecular dissection revealed numerous attachment sites, a finding that may be related to the flexibility of [^{3}H]-azidopine.

P-gp was also photo-labelled with [^{3}H]-azidopine in the absence or presence of other substrates or inhibitors. [^{3}H]-azidopine labels two distinct locations previously identified in P-gp: the site within the carboxy half and the one within the amino half between residues 198 and 440 (Bruggemann et al. 1992). Vinblastine strongly inhibited azidopine labelling of P-gp at both photo-labelling sites and the authors suggested that this supported the existence of a common binding site for azidopine and vinblastine. Given the subsequent investigations with equilibrium binding assays (Sect. "Classical Receptor-Drug Analysis of Multidrug Binding to P-gp"), the data are more likely explained by the presence of multiple binding sites linked by a negative heterotropic allostery (Ferry et al. 1992, 1995; Martin et al. 1997). These two investigations clearly demonstrated an allosteric interaction between vinblastine and several 1,4-dihydropyridines; the latter including azidopine.

To determine the binding site of paclitaxel, Wu et al. developed a photo-affinity analogue bearing a 3′-BzDC group and used site-directed antibodies to identify the labelled residues (Wu et al. 1998). The domain mapping identified residues 985–1088 in mouse mdr1b P-gp, which includes half of TM12 and terminates immediately after the Walker A motif of NBD2. Another study using a 7-DzDC group photo-affinity analogue of paclitaxel highlighted the importance of residues 683–760, a region that includes TM7 and half of TM8. In a third investigation, Safa et al. used the analogue [^{125}I]-NAST to localise the

paclitaxel-binding sites (Safa 2004). The sites were identified with site-directed antipeptide antisera raised against ten different domains. Following labelling, protease digestion revealed four major photo-labelled fragments of 12, 10, 8 and 6.5 kDa, which were immunoprecipitated by an antibody directed against amino-acid residues N-terminal to TM3. A short, five amino-acid intracellular sequence that was C-terminal to TM4 was found to be common across all these peptides. [^{125}I]-NAST was found to be associated with another 6.5-kDa peptide that located C-terminal to TM segment 6. The data suggests an overlap in the binding domain of paclitaxel with vinblastine, verapamil and azidopine (Safa 2004) that includes amino-acid residues in TM segments 4 and 6.

It appears that three investigations using photo-active analogues of paclitaxel label P-gp at three distinct sites. A possible interpretation is that all the three regions comprise the paclitaxel-binding site and that they are proximal in the 3-D structure of P-gp. However, the orientation of the photo-active moiety on paclitaxel will dictate which of the regions is labelled. In addition, the photo-active moiety may be located on a highly flexible region of the probe and that regions distal to the "true" binding site are labelled (Glossmann et al. 1987).

P-gp was labelled with [^{125}I]-IAAP, a photo-affinity analogue of the modulator prazosin (Dey et al. 1997) and subsequently subjected to proteolytic cleavage with trypsin to separate the two halves of P-gp. The ratio of [^{125}I]-IAAP bound between the N-terminal and C-terminal halves of the P-gp molecule was 2:3. The effect of *cis*(Z)-flupentixol, an antipsychotic modulator of P-gp, on labelling of the two halves by [^{125}I]-IAAP was also measured. There was an approximately 10-fold increase in photo-labelling of [^{125}I]-IAAP at the C-terminal half of P-gp with no change at the N-site. This supported two separate "events" of drug interaction rather than simply two covalent attachment sites for [^{125}I]-IAAP within a single site. Conceivably, the two photo-labelled sites are spatially distinct substrate interaction pockets that exist within a larger drug-binding domain. In addition, the concentrations of vinblastine and cyclosporin A required to inhibit [^{125}I]-IAAP photo-labelling at the C-terminal site were increased 5–6-fold in the presence of *cis* (Z)-flupentixol. In contrast, the potency for reducing [^{125}I]-IAAP labelling at the N-terminal site was unaffected by *cis*(Z)-flupentixol. The photo-labelling of the N-terminal site by [^{125}I]-IAAP was also less susceptible to vanadate trapping of P-gp. This study supports the existence of at least two non-identical substrate interaction sites in P-gp, at least [^{125}I]-IAAP (Dey et al. 1997).

Finally, these early attempts at locating the drug-binding sites suffered from an inability to generate precise and small fragments of P-gp and could thereby only proffer regions of interaction. However, collectively the data supported the hypotheses that P-gp contained a large binding domain comprising segments from multiple regions/helices.

Enhanced Detection of Photo-Affinity Labelled Regions of P-gp

Several novel photo-affinity ligands generated from propafenone were used to photo-label P-gp, aiming to identify the binding site for drugs (Ecker et al. 2002; Pleban 2004). Propafenones were previously shown to inhibit the transport of the P-gp substrates rhodamine 123, doxorubicin, vinblastine and mitoxantrone (Chiba et al. 1995; Ecker et al. 1996). In addition, a radioactive propafenone analogue [^{3}H]-GPV51 was used to specifically photo-label plasma membrane vesicles expressing P-gp, confirming its direct interaction with the transporter. Following photo-labelling with [^{3}H]-GPV51, membrane proteins were separated by SDS–PAGE and visualised by silver staining. The core-glycosylated 140 kDa P-gp band was excised and proteolytically degraded by chymotrypsin "*in-gel*". The fragments were eluted from the gel and the ligand-labelled peptides identified with matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry (Ecker et al. 2002). This procedure gave considerable advantages to the earlier studies involving antibody precipitation. First, the exact peptide and amino-acid residues could be identified and second, the coverage of peptide fragments was ~90 % in the TMDs and ~80 % for the NBDs. The mass-spectrometry data identified significant [^{3}H]-GPV51 labelling in TM helices 3, 5, 8 and 11, which, like the antibody studies, suggests the binding site comprises multiple protein segments.

Another investigation by the team (Pleban 2004) made use of the emerging structures of ABC proteins, in particular, the MsbA lipid transporter from *Vibrio cholera* (Chang 2003). The MsbA structure was used as a template to produce a homology model for P-gp. The homology model predicts that the helices labelled by [^{3}H]-GPV51 lie at the interfacial regions between the two transmembrane domains of P-gp. One interface comprises TM3/11 and the other TM5/8. Although this homology model was eventually proven inappropriate due to retraction of the original structure, the interfacial location of the helices remains a valid assumption. The involvement of these helices has been borne out with models of P-gp based on the structure of Sav1866, mouse P-gp and a *C. elegans* homologue of P-gp.

An interpretation of this key finding was that the interfaces (TM3/11 and TM5/8) operate as a "gate" for substrates to enter the large binding domain in the central cavity (Pleban 2004; Crowley and Callaghan 2010). This interpretation is in broad agreement with results from double-cysteine mutant cross-linking studies (Loo and Clarke 2005). The findings are also compatible with both simultaneous binding of drugs on P-gp. Moreover, the gate model implies that any drug will bind sequentially at the interface and then within the central binding domain. However, there is no evidence from equilibrium binding studies for a two-component binding isotherm to describe drug interaction with P-gp. Potentially, the interfaces are not gates for entry to the cavity, but may exist as allosteric modulatory sites on the protein.

Multiple Fluorescence-Based Strategies to Locate Drug-Binding Sites

Spectroscopic techniques provide a key experimental tool for molecular biochemists and reveal an astonishing amount of information on protein structure and function. Fluorescence spectroscopy is widely used due to its general accessibility and the array of compounds and applications available. The technique has been widely used to facilitate our understanding of the molecular mechanism of P-gp. The field has taken advantage of the number of fluorescent substrates of P-gp, the presence of intrinsic tryptophan residues in key locations and the ability to attach fluorophores to target regions of the protein. Fluorescence spectroscopy has provided modest advances in our search to identify the location of binding sites on P-gp, but it has generated a wealth of functional data. In particular, the approach has informed on the nature of bioenergetic coupling in P-gp and the conformational changes that underlie its complex mechanism of drug translocation.

Fluorescence Quenching of Covalently Attached Probes to Characterise Drug Binding

The team headed by Frances Sharom developed a fluorescence quenching technique to investigate the direct binding of substrates and inhibitors of P-gp. One of these fluorescence techniques involves the use of the sulphydryl-reactive fluorescent probe, 2-4′-maleimidylanilino-naphthalene-6-sulphonic acid (MIANS) for site-directed labelling (Liu and Sharom 1996, 1997; Qu and Sharom 2001). In particular, MIANS has been shown to preferentially form a covalent attachment to P-gp at the cysteine residue located in the Walker A motif of each NBD. MIANS attached at the NBDs display a saturable quenching of its fluorescence upon binding of ATP, adenosine diphosphate (ADP) or its non-hydrolysable analogues. The binding of the nucleotide likely affects the local environment of the MIANS group which is located close to the ATP-binding sites in the NBDs. This results in the fluorescence quenching observed as a decrease in the emission intensity and/or the maximum emission wavelength. This approach has demonstrated the binding affinity of ATP/ADP, the co-operativity between NBDs, the ability of P-gp to bind two ATP molecules and the kinetics of ATP hydrolysis (Liu and Sharom 1997; Qu and Sharom 2001).

Quenching of MIANS fluorescence has also been demonstrated in response to the binding of P-gp substrates and modulators. The quenching profile follows a saturable and hyperbolic relationship with drug concentration, which enables an indirect estimation of an affinity for the interaction with drug at the TMDs (Liu and Sharom 1996). It is important to note that the affinity comprises two events; initial drug binding to P-gp and a component consisting of the conformational change required to alter the fluorescent signal. Therefore, the affinity from such measurements may only be considered an apparent K_D value. Nonetheless, the apparent K_D

for MIANS quenching has been determined for over 80 structurally different P-gp drugs with values ranging from 25 nM to 260 μM, demonstrating that P-gp differentially binds substrates with an affinity over four orders of magnitude (Sharom 1997).

This long-range crosstalk between the TMDs where drugs bind, and the NBDs, where the MIANS is attached, demonstrates coupling between the two domains (Liu and Sharom 1996). Moreover, this inter-domain coupling, and subsequent conformational change, is a critical step in the ability of substrates to stimulate ATPase activity. In addition, the presence of bound drug reduces the rate and extent of MIANS labelling of P-gp, which is further proof of the specific conformation changes elicited in the NBDs. Numerous investigations have demonstrated communication in the opposite direction and the process of drug translocation is considered to be driven by the steps involved in ATP hydrolysis. Clearly, this communication occurs in both directions and is consistent with a high level of coupling.

Fluorescence of Intrinsic Tryptophan Residues to Characterise Drug Binding

Another approach to investigate binding makes use of the intrinsic fluorescence of P-gp afforded by its tryptophan residues. Both human and hamster P-gp isoforms contain 11 intrinsic tryptophan residues that are distributed throughout the protein. In addition, the tryptophan residues are highly conserved across the P-gp family and an involvement in substrate interaction and recognition has been suggested given the hydrophobic nature of substrates (Liu et al. 2000). Hydrophobic substrates such as vinblastine or rhodamine 123 contain multiple aromatic rings and these undertake π–π stacking interactions with the side chains of aromatic-rich TM helices. These stacking interactions with tryptophan residues will alter the spectral properties of the latter and may provide evidence for binding of drug to the protein (Fig. 2). Interestingly, there are several tryptophan residues (and other aromatic residues) in TM3/11 and TM5/8, located at the TMD/TMD interface (Pawagi et al. 1994). As described in Sect. "Enhanced Detection of Photo-Affinity Labelled Regions of P-gp", these helices mediate the binding of several substrates/modulators of P-gp. Consequently, drug binding to P-gp in these helices may directly alter tryptophan fluorescence, rendering the assay a useful reporter for binding.

One of the advantages of fluorescence spectroscopy is the intimate relationship between the fluorescent profile and its local physical and chemical environment. Consequently, tryptophan fluorescence kinetics may reveal whether the residue is subject to any steric hindrance to its motion and report changes in the polarity of the local environment. Measurement of fluorescence lifetime for the intrinsic tryptophan residues revealed the presence of two components (Lugo and Sharom 2009). The fast component is solvent exposed (i.e. aqueous) and likely to be located in exposed cytoplasmic loops. The location of this fast component in an aqueous

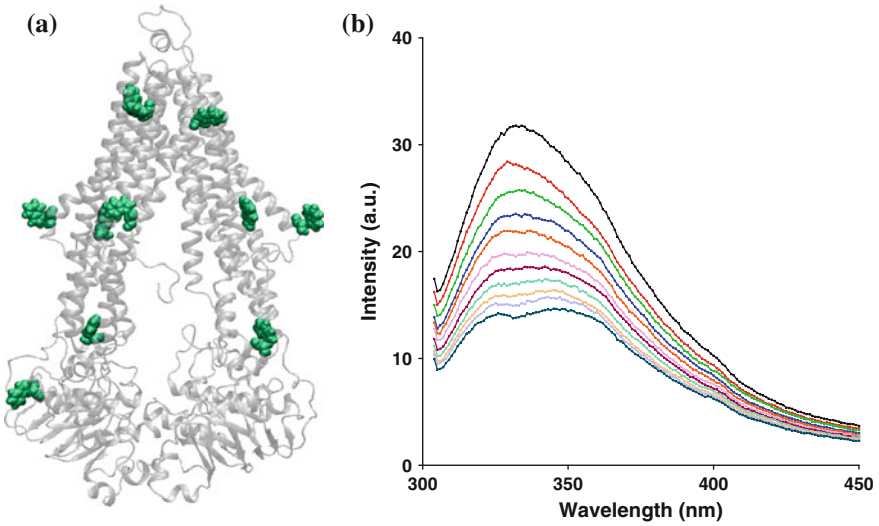

Fig. 2 Using intrinsic tryptophan fluorescence to identify substrate-binding sites. **a** The molecular dynamic (MD) equilibrated P-gp model indicating the 11 intrinsic tryptophan residues. Quenching (decrease in intensity) of the fluorescent spectrum profile of P-gp in the presence of particular substrates can indicate drug binding to the protein. Binding results in a conformational change which likely alters the environment of the tryptophan residues changing the fluorescent profile. **b** A representative tryptophan fluorescence spectrum profile of intensity as a function of P-gp substrate concentration

environment was demonstrated by the use of various hydrophobic and hydrophilic quenching agents (e.g. acrylamide, iodide, benzene) to gauge their relative effects on fluorescence (Liu et al. 2000; Lugo and Sharom 2005). The quenching profile and the emission maximum for fluorescence suggested that the tryptophan residues located in a polar environment did not appear to contribute to the observed fluorescence. This may suggest that the tryptophans residing in an aqueous region are located within a very tightly folded domain of the protein, or that they are internally quenched (Liu et al. 2000).

The slow component of the tryptophan fluorescence decay is localised within the lipid membrane; again verified with the use of a number of chemical quenchers. In addition, the fluorescent drug LDS-751 affects the slow component of tryptophan fluorescence decay, which is achieved due to either direct intercalation with the amino-acid or via allosteric conformational changes.

Fluorescent P-gp Substrates and the Kinetics of Transport

P-gp mediated transport of the fluorescent substrate Hoechst 33342 was measured in plasma membrane vesicles isolated from Chinese hamster ovary cells (CH^RB30).

When sequestered in the lipid environment of the membrane, the fluorescence of Hoechst 33342 is enhanced and then lost upon moving into aqueous solution. Transport by P-gp in the inside-out plasma membrane vesicles was initiated following addition of Mg·ATP and observed as a reduction in fluorescence (Shapiro and Ling 1995). This assay system enables continuous fluorescent monitoring, thereby allowing accurate measurement of transport rates. The kinetics of Hoechst 33342 transport was measured in the presence, or absence, of another fluorescent P-gp substrate, rhodamine 123 (Shapiro and Ling 1997b). Conversely, the transport of rhodamine 123 was also measured in the P-gp-rich vesicles in the presence or absence of Hoechst 33342. Each substrate was able to stimulate the transport of the other, which is not possible if they interacted at a single binding site; such an interaction would have resulted in one substrate competitively inhibiting transport of the other. Both dyes were transported simultaneously and the stimulatory effect could not be attributed to the presence of one site for transport and the other for allosteric regulation. The positively co-operative manner in which each substrate stimulated the transport of the other is best explained by the presence of at least two transport-competent drug-binding sites on P-gp.

This is further supported by the observation that colchicine and quercetin stimulated the transport of rhodamine 123, but inhibited the transport of Hoechst 33342 (Shapiro and Ling 1997a). In contrast, the anthracyclines doxorubicin and daunorubicin inhibited rhodamine 123 transport, but stimulated Hoechst 33342 transport. The two sites were designated the H-site which preferentially binds and transports Hoechst 33342, quercetin and colchicine, and the R-site, which is the preferential binding site for rhodamine 123 and the anthracyclines. Overlapping specificity of these two distinct sites has been suggested for some drugs, given that vinblastine, actinomycin D and etoposide were all able to inhibit the transport of both dyes (Shapiro and Ling 1997a, b).

A flow-through system was used to measure daunorubicin efflux or accumulation from P-gp expressing cells and represented one of the only documented reports showing that the transporter works against a considerable concentration gradient (Lankelma et al. 1990). In a subsequent investigation, the interaction between the transported substrate daunorubicin and the modulator verapamil was examined (Spoelstra et al. 1994). At low concentrations of daunorubicin, verapamil caused a non-competitive inhibition of its transport; however, at high substrate concentration this inhibition became competitive. This curious finding can be explained with a model of P-gp wherein daunorubicin may bind at two pharmacological sites with different affinities. At low daunorubicin concentrations it bound at the high affinity site and the effects of verapamil on transport kinetics occurred through an allosterically linked process (i.e. non-competitive). However, daunorubicin binds to both the high and low affinity sites on P-gp at high concentrations. The low affinity site is also the location for verapamil binding. Consequently, under these conditions the verapamil effect on daunorubicin transport was partly due to a competitive interaction. Similar observations have been described for the interaction between anthracyclines and the transport of Hoechst 33342 (Shapiro and Ling 1997b). At lower concentrations, the anthracyclines bound at the R-site, which is linked to the

H-site (Hoechst 33342) by a positive heterotropic allostery. When administered at higher concentrations, the anthracyclines also competed for binding with Hoechst 33342 at the H-site; thereby inhibiting its transport.

Further studies with the fluorescent substrates and modulators discovered the existence of a third binding site on P-gp (Shapiro et al. 1999). This was termed the P-site by virtue of the fact that prazosin and progesterone both interacted at this pharmacological location. Previous investigations had suggested that although progesterone was capable of modulating the transport function of P-gp, it was unlikely to be a substrate (Ueda et al. 1992). Prazosin and its analogues had been previously demonstrated to bind directly to P-gp, stimulate its rate of ATP hydrolysis, and alter substrate transport rates; however, it was classified as a weak transport substrate, or a modulator (Greenberger 1993). Consequently, it was suggested that the P-site acts primarily as an allosteric site and that compounds bound to it are not transported. In support of this, it was shown that drug binding to the P- and R-sites stimulated the transport of Hoechst 33342. In contrast, binding to the P-site alone stimulates the transport of either Hoechst 33342 or rhodamine 123. Finally, binding of drug to both the H- and P-sites appears to stimulate rhodamine 123 transport, although the effect is lower than that produced by binding to the sites individually. However, later studies using BODIPY-FL-prazosin indicated that this fluorescent derivative was a substrate for P-gp mediated transport (Gribar et al. 2000). Consequently, the P-site should be reclassified to a transport-competent one, although non-transported modulators (e.g. progesterone) were also capable of interaction (Shapiro et al. 1999).

These kinetic investigations with fluorescent substrates not only defined the existence of multiple substrate transport sites, but they also revealed complex allosteric interactions and the possibility that drugs may bind at more than one site on P-gp. Multiple factors such as this can synergise to produce the renowned substrate promiscuity of P-gp.

Fluorescence Resonance Energy Transfer to Characterise Drug Binding

Fluorescence resonance energy transfer (FRET), which is the transfer of energy from a fluorescent donor to the excited state of an acceptor fluorophore, depends on the distance between the two and only occurs if separated by 10–75 Å. This enables the technique to be used as a ‘spectroscopic ruler’ measuring intra- and inter-molecular distance.

The fluorescent probe 4-Chloro-7-Nitrobenz-2-Oxa-1,3-Diazole (NBD-Cl) was inferred to covalently attach to Cys428 and Cys1071, both of which are in the catalytic site of P-gp (Qu and Sharom 2001, 2002). Labelling at these two sites facilitates the examination of inter-domain communication in P-gp and the use of FRET to define intra-molecular distances. The fluorescent substrate H33342 bound

to P-gp and exhibited an increase in fluorescence intensity, coupled with a large blue shift in its emission maximum (i.e. lower λ_{max}), indicating a non-polar environment. The spectral properties of H33342 render it a FRET donor to NBD-Cl and this was demonstrated as a marked reduction in its emission intensity. The degree of FRET was used to estimate a distance of 38 Å between the bound H33342 and the NBD-Cl bound at the catalytic site of P-gp (Qu and Sharom 2002). H33342 binds to the "H-site" of P-gp (Shapiro and Ling 1997b) (Sect. "Fluorescent P-gp Substrates and the Kinetics of Transport"), and the FRET data also indicates that this site is found 10–14 Å below the external surface of the membrane, within the cytoplasmic leaflet (Qu and Sharom 2002).

A FRET strategy was also used to identify a broad location for the "R-site" of drug binding on P-gp (Shapiro and Ling 1998). The ligand for the "R-site" was LDS-751 and it displayed a marked increase in the fluorescence intensity on binding to P-gp, indicative of a hydrophobic environment. LDS-751 was chosen as the "R-site" ligand since it is a FRET partner for the intrinsic tryptophan residues and it caused a 40 % reduction in the fluorescence of the latter. FRET was proposed to occur with the cytosolic, solvent exposed tryptophan residues and an approximate location for the "R-site" was also placed in the cytoplasmic leaflet of the membrane. That the location of both the "R- and S-sites" is found in the cytoplasmic leaflet of the bilayer was used to support a role for P-gp as a flippase that translocates lipids between leaflets of the bilayer.

Although not a FRET interaction, there was also a spectral interaction between H33342 and LDS-751 upon binding to P-gp, which again demonstrates that the protein is capable of binding more than one substrate or modulator simultaneously. This observation is in agreement with the photo-affinity and mutagenesis data presented in previous sections. It also highlights the utility of biochemical approaches and how molecular understanding can be reached with a combination of approaches. Moreover, the investigations with fluorescence spectroscopy have provided not only information on the location of binding sites, but the properties and characteristics of them.

Can Structural Information Locate Drug-Binding Sites?

The preceding sections have detailed how our understanding of the nature of drug binding to P-gp, and the location of this binding, has improved in the last three decades. The availability of structural data would boost our understanding further and reconcile some of the contradictory observations from biochemical and biophysical studies. In order to reveal the sites of drug binding, this structural data will need to be obtained in the presence of bound substrate. Furthermore, provision of data to fully describe the mechanism of drug translocation will require structural information in multiple conformations such as nucleotide-free, nucleotide-bound, post-hydrolytic and post-dissociation of drug. In order to reveal the chemical nature

of drug interaction with the protein (i.e. hydrogen bonding versus π–π stacking) we will need structural data at high resolution; near or below the 2 Å level.

Overview: Structure of P-gp

The availability of purification procedures that provided high yields of fully functional P-gp begun to surface in the mid-1990s, although they used classical multiple step chromatographic methods (Liu and Sharom 1996; Callaghan et al. 1997). The use of affinity tags (i.e. polyhistidine) from the late 1990s improved the quality and purity of the protein, and the ease of its purification (Lerner-Marmarosh et al. 1999; Taylor et al. 2001; Loo and Clarke 1995b). The first attempts at obtaining structural data for P-gp also begun in earnest soon after these milestones. Initial attempts used electron microscopy (EM) with single particle analysis (SPA) and eventually progressed to 2-D crystals in a membrane environment. It was not until 2009 that the first structure of P-gp (mouse) using an X-ray crystallography approach was published (Aller et al. 2009), following considerable effort by multiple teams in the field. The structure (3.8 Å resolution) continues to generate debate amongst researchers and has now undergone rounds of refinement and reinterpretation. Another structure (Jin et al. 2012), of a P-gp homologue in *C. elegans,* has been obtained to higher resolution (3.4 Å), however it was in an identical conformation and generated in the absence of bound substrate/modulator. Progress has been steady and the information has illuminated our understanding of drug binding to the protein. However, like most scientific endeavours, it has raised several new questions.

The First Structure of P-gp: Electron Microscopy

The first structure of any full-length ABC protein was obtained in 1997 (Rosenberg et al. 1997) using electron microscopy-single-particle analysis (EM-SPA) of hamster P-gp purified from drug selected CH^RB30 cells (Callaghan et al. 1997). The structure was obtained to 2.5 nm (25 Å) resolution and the SPA technique was done with P-gp in detergent micelles. The resolution of EM-SPA will not provide high resolution for a protein of this size and was intended merely to generate a preliminary view of the gross organisation and dimensions of P-gp. The protein displayed a cylindrical geometry with a diameter of 10 nm and a height of 8 nm. Given that the average height of a bilayer is $\sim$4 nm, the authors posited that half of the structure in the "vertical" plane was found within the membrane. Based on the dimensions of other proteins and the size of membrane spanning helices, it appeared that the TMD of P-gp was somewhat loosely packed. Perhaps the standout feature, and at the time the most controversial one, was the central pore of diameter $\sim$5 nm. Based on the staining properties of the particles it was suggested that the

pore was aqueous. Lectin-gold labelling of the extensively glycosylated hamster P-gp isoform was used to orientate the particles and thereby identify the NBDs. The central "aqueous" pore of the P-gp particles was suggested to be open to the extracellular environment. The final observation from this structure was that the TMDs also contained a discontinuity (or gap) within the plane of the membrane that may provide access for substrates to the central cavity. The latter point was intentionally speculative, but it is in agreement with the photo-affinity/MALDI-TOF data proposing that P-gp contains gates for substrates (Ecker et al. 2002).

Gradual Evolution of the EM-Derived Structure of P-glycoprotein

The structural efforts with EM evolved to higher resolution over the next few years and the next data was obtained for 2-D crystals of P-gp, reconstituted into membranes (Rosenberg et al. 2001). The key feature of this manuscript was the presentation of data for P-gp trapped in multiple conformations; namely AMP-PNP bound (i.e. nucleotide bound) and ADP.Vi trapped (i.e. post-hydrolytic). Comparison of the TMD regions revealed extensive conformational transitions during passage between the three catalytic states. This observation would be the spur for numerous pharmacology studies on the effects of events at the NBDs on drug binding. Moreover, it provided direct evidence that events at the NBDs instigate the major conformational transitions essential to the drug translocation process. A subsequent publication obtained resolution improvements and focussed on comparison between the nucleotide-free and -bound conformations (Rosenberg et al. 2003). The investigation provided confirmation of the central pore with dimensions of 5–6 nm in diameter and 5 nm depth. The central pore was open to the extracellular space and closure at the cytoplasmic face was produced by the NBDs. Significant structural transformation occurs in this region in the presence of nucleotide, and the TM helices rearrange to support a "helix rotation" motion. This results in the opening of the internal cavity along the length of the membrane, which may enable the entry of hydrophobic drugs to the putative drug-binding pocket.

The final instalment of EM-based structures for P-gp was resolved to approximately 8 Å for protein trapped in the nucleotide-bound state (Rosenberg et al. 2005). The structure was obtained from cryo-EM of 2-D crystals of P-gp to provide further resolution of the overall topography and packing of the transmembrane helices. The structure displayed a pseudo-2-fold symmetry within two blocks of six TM helices, showing the presence of the 12 putative membrane spanning helices and some evidence of inter-domain cross-over facilitated by the sixth helix in each domain. With the integration of available biochemical data, the authors speculated the location of numerous TM helices. However, due to the low resolution, definite identifications could not be assigned. Although only limited predictions of the

location of helices were possible from the structure, the comparison between conformations of P-gp provided crucial information on the molecular mechanism of drug translocation.

Crystal Structure of a Nucleotide-Bound P-gp Homologue; Sav1866

As is often the case in structural biology, structures are obtained from some unlikely sources and often for uncharacterised proteins. In keeping with this theme, the first full-length structure generated using X-ray crystallography for an ABC export pump was obtained for the *S. aureus* exporter Sav1866 (Dawson and Locher 2006). Little was known about this pump prior to the structure and ensuing biochemical studies confirmed it was a multidrug efflux pump. The structure was obtained to 3.0 Å and contained 12 transmembrane helices, six from each of the monomers. Unlike the import proteins, there was considerable cross-over of TM helices between the two monomers in the structure. The structure was obtained in the absence of any bound substrate, however successful crystallisation was obtained in the presence of ADP. Consequently, the two NBDs were arranged in close apposition in the standard "sandwich dimer" configuration of the Walker A/B and signature motifs. This post-hydrolytic structure also contained a central aqueous cavity in an externally facing configuration.

The same research team published a subsequent structure for Sav1866 (3.4 Å) determined in the presence of AMP-PNP, a non-hydrolysable analogue of ATP (Dawson and Locher 2007). The latter structure was obtained to investigate the physiological relevance of the earlier structure, as in the ADP-bound state the dimerised half-transporter, adopted an outward-facing conformation (Dawson and Locher 2006). The apposition and arrangement of the NBDs was similar between the two structures and the structure obtained in the presence of AMP-PNP also showed an outward-facing conformation with the central cavity open to the extracellular surface of the membrane. The outward-facing configuration subsequent to nucleotide binding or hydrolysis is substantiated by considerable evidence with ABC proteins. However, the lack of difference between the AMP-PNP and ADP-bound states is more puzzling given the reported differences with P-gp (Rosenberg et al. 2001; Martin et al. 2001). It may transpire that only following dissociation of ADP will the protein adopt an inward-facing conformation, exposing a central cavity (binding site) to scan the inner leaflet of the lipid bilayer and bind substrates with high affinity (Higgins and Linton 2004; Smith et al. 2002). That bound ADP was sufficient for the transporter to adopt the outward-facing conformation was interpreted to suggest that the detergent solution, (in which the protein was obtained), shifted the conformational equilibrium of the protein and may not reflect in vivo conditions. The crystal structure provided a molecular basis

for Sav1866 mechanism as it clearly showed a coupling between the ATP-bound state and drug release as a result of the outward conformation.

X-Ray Crystal Structure of Mouse P-gp

In 2009 the first X-ray crystal-based structure of a mammalian ABC transporter, namely mouse P-gp, was resolved to 3.8–4.4 Å (Aller et al. 2009). Mouse P-gp has 87 % sequence identity to human P-gp and from a pharmacological perspective is indistinguishable from the human isoform (Taylor et al. 1999). Three distinct structures were solved in the publication and included one in the absence of nucleotide/drug, and two structures in the absence of nucleotide, but in the presence of two cyclic hexapeptide stereoisomers. The presence of bound drug was greeted with enthusiasm for its potential to provide a molecular basis for substrate promiscuity of the transporter; however, the resolution remains short of atomic level detail.

The P-gp structure, obtained in the absence of both nucleotide and substrate or inhibitor, showed an inward-facing conformation resolved to 3.8 Å (PDB 3G5U). The model was generated using multiwavelength anomalous dispersion mapping. A striking feature, and one that has engendered considerable debate (Gottesman et al. 2009; Li et al. 2014), is the physical separation of the two NBDs by a distance of 30 Å. It remains unclear if this is the "native" configuration of empty P-gp, or whether it is an artefact of crystallisation. The latter is likely given that protein is in detergent micelles (i.e. relaxed) and the crystal unit–unit contact is conferred by the NBDs. Due to the large separation of NBDs, the two blocks of TMs were observed to adopt a conformation forming a large internal cavity. The presence of a central cavity is in agreement with previous structural and biochemical data, albeit with different dimensions. The characteristic "domain swapping" of helices was consistent with that first documented for Sav1866. Moreover, the central cavity is open to the cytoplasmic face of the membrane, but closed to the extracellular surface by TM helices. The volume of the pocket within the central cavity was estimated at 6000 $Å^3$. This is an extraordinary dimension that renders it capable of facilitating the simultaneous binding of drugs at different regions within the cavity. This is evident by the molecular surface area of established P-gp substrates; vinblastine 154 $Å^2$, paclitaxel 221 $Å^2$, and doxorubicin 206 $Å^2$. It was also suggested that two pairs of TM helices may form putative portals in the inner leaflet of the membrane to enable substrate passage from the lipid bilayer into the central pore. Furthermore, solvent accessible amino-acid residues lining the internal cavity were investigated and showed the majority of the residues contributing to binding of the cyclic peptides are hydrophobic and aromatic (58 of the 73 solvent accessible residues). Within the drug-binding pocket, it was found that hydrophobic and aromatic residues are present at the upper (extracellular) region, whereas polar and charged residues are more frequent in the lower or cytoplasmic region. This supports data obtained for the topology of TM6/12, both of which contribute to cavity lining (Crowley et al. 2010b; Rothnie et al. 2004; Storm et al. 2008).

Unfortunately, it appears that the mouse P-gp could not co-crystallise in the presence of any of the well-characterised arrays of substrates and modulators. The authors did have success in co-crystallising P-gp with two stereoisomeric cyclic peptide inhibitors, cyclic-*tris*-(R)-valineselenazole (QZ59-RRR) and cyclic-*tris*-(S)-valineselenazole (QZ59-SSS). This was the first report using these two uncharacterised compounds that appear to act as inhibitors, or modulators, of P-gp. It is unclear if they are substrates for transport and whether their binding overlaps with any of the established substrates/modulators. Co-crystallised P-gp adopted the expected inward-facing conformation and the stereoisomers surprisingly appeared to bind at distinct sites. QZ59-RRR was observed to bind in the "centre of the transporter" and may interact with TMs 1, 5, 6, 7, 11 and 12. Conversely, QZ59-SSS simultaneously binds at two distinct sites at the "upper" and "lower" regions of the central cavity facilitated by TMs 1, 2, 6, 7, 11 and 12. A portion of QZ59-SSS at the upper site was observed in a disordered state and there is no available explanation for this. At the resolution obtained for the structure, assigning directly interacting residues or the points of interaction between peptide and drug remains speculative. There was high structural similarity of crystallised P-gp in the absence of inhibitor, co-crystallised with QZ59-RRR or co-crystallised with two molecules of QZ59-SSS. This is in conflict with the proposed induced fit mechanism for substrate transport by P-gp, which would cause conformational changes upon drug binding. Biochemical data will be essential to ascertain stoichiometry of binding and to elucidate which residues directly interact with the cyclic peptides and more established substrates and inhibitors.

In 2013, three crystal structures of mouse P-gp were published (Ward et al. 2013) (see Sect. "Crystal Structure of a P-gp Homologue from C. elegans") in a follow-up study, and in response to the structure obtained from *C. elegans* (Jin et al. 2012). All the structures were in the inward-facing conformation and obtained in the absence of nucleotide. Two of the structures were crystallised in the absence of inhibitors, however one was obtained in the presence of a nanobody bound to the C-terminal side of NDB1, preventing the dimerisation of the NBDs and thereby inhibiting ATP hydrolysis of the protein. The three structures presented different separation distances between the NBDs, which was suggested as proof of conformational flexibility of the inward-facing conformation of P-gp. In particular, two crystal structures (in the absence of inhibitor or drug) diffracted to 3.8 and 4.0 Å, corresponding to NBD separations of 31 and 36 Å, respectively; which is different to the original structure by the consortium. The final structure was co-crystallisation with a nanobody inhibitor Nb592 to a resolution of 4.1 Å and an NBD separation of 30 Å. It was suggested that the observed conformational changes to the presence of a "TM-hinge region" formed by extracellular loops between the TM helices. The conformational flexibility of the inward-facing conformation, most drastically noticed in the cytosolic domains, is therefore a consequence of small movement at this pivot point. Another highly plausible interpretation is that the use of detergent micelles enables considerable conformational freedom compared to the more rigid environment of a phospholipid bilayer. In fact, the literature is replete with

biochemical studies demonstrating the difference in activity and functional properties of P-gp between lipid- and detergent-based milieu.

Crystal Structure of a P-gp Homologue from C. Elegans

Shortly following the publication of the mouse P-gp crystal structure, the X-ray crystallographic derived structure of a P-gp homologue from *Caenorhabditis elegans* was resolved to 3.4 Å (PDB 4KSB) (Jin et al. 2012). The P-gp structure from *C. elegans* was obtained in the absence of nucleotide or drug, and with the transporter in an inward-facing conformation. The substrate profile and amino-acid sequence vary considerably between *C. elegans* and mammalian P-gp, with only 46 % identity to the murine isoform. However, the authors argue that that structural information from the *C. elegans* isoform can provide mechanistic insight into the function of the protein.

The *C. elegans* version of the structure of P-gp displayed separation between the NDBs of 53 Å, which is considerably larger than that observed in the crystal structures of mouse P-gp (Aller et al. 2009; Ward et al. 2013). Another difference was that the structure contained only one portal formed by the TMs and a discontinuity in the helical structure of TM10/12, which line the portal. The greater preponderance of disordered loops in this region was thought to increase the number of contact points, or interactions, between drugs and amino-acid residue.

The interactions between TMDs and NBDs are observed in this structure with four intracellular helices (IH1–IH4), providing a domain interface that may be involved in communication. To further support the significance of this interface, the sequence identity between amino-acid residues in this region for *C. elegans* and human P-gp was high. Additionally, a highly conserved collection of amino acids in IH1, IH4 and NBD1 were proposed to form a salt bridge at the TMD–NBD1 interface. A similar structure was observed in the TMD–NBD2 interface, with conserved residues in IH2, IH3 and NDB2.

The crystal structure of *C. elegans* P-gp, and of the murine isoforms, has provided a wealth of information on the functional organisation of the transporter, tantalising glimpses into the drug-binding domain/sites and testable hypotheses regarding the inter-domain communication that transduces drug binding to provision of energy.

Bioinformatics Approaches to Reveal Drug-Binding Sites

The generation of a high-resolution protein structure should not be considered the closure of a research field; it is more often the instigator of considerable further investigation. The structures of helices, sheets and loops, that we view, are actually models built from the primary data—i.e. X-ray diffraction patterns. Advances in

bioinformatics, in particular protein molecular modelling, enable rigorous interpretation of the structures, their stability and facilitates their refinement. Currently, we do not have structural data of sufficient resolution to unequivocally determine which residues are involved in mediating drug–P-gp contact. Molecular modelling, drug docking and further biochemical data, will build on the solid foundation of structural data to provide this. The following sections describe how bioinformatics and biophysical approaches have begun to unravel the mysteries of drug binding to P-gp.

Human P-gp Homology Modelling

Following the release of Sav1866 X-ray structure in 2006, O'Mara and Tieleman investigated the suitability of this template for homology modelling. Homology models of P-gp were generated to represent different stages in the transport cycle; namely, closed and open (O'Mara and Tieleman 2007). The ADP-bound Sav1866 structure was used to render the closed state (i.e. nucleotide bound). To produce a model in the absence of nucleotide (i.e. open), insights were used from the importer protein BtuCD and the NBDs from the maltose importer (i.e. MalK subunits). The open state model of P-gp was verified for orientation and energy minimisation. Significant conformational changes were observed in the TMDs that altered the geometry and residue distribution of the central cavity. The authors reported a rotation of the TM helices to re-orientate polar residues lining the central cavity in the closed state to inter-helical regions, thereby exposing hydrophobic residues. This supports previous data of the closed state representing a low affinity extrusion pore that becomes a high affinity binding site for recognition and transport of substrates (Dawson and Locher 2006).

Another study, by Ravna et al. also investigated the use of Sav1866 as a template for the structure of P-gp (Ravna et al. 2007). This model strongly supported biochemical data that attributed specific TM helices to drug binding since the orientation and nature of the residues lining the central cavity would support the interaction of hydrophobic substrates. The authors reported that although the sequence identity between Sav1866 and P-gp was only 31 %, the conserved secondary structures and overall geometry between the two proteins suggest that the Sav1866 may be a realistic template for the structure.

These fledgling attempts at providing molecular models of P-gp structure were used in a number of publications to facilitate reconciliation of biochemical data from a structural perspective (Crowley et al. 2009, 2010b; Storm et al. 2007, 2008). This strategy was vital in not only interpretation of results, but it provided a focus or direction for subsequent mutagenesis studies, and therein lies the true value of the technique. Subsequently, a molecular model of human P-gp was generated from the murine structure and the effects of ATP and Mg^{2+} binding were investigated (O'Mara and Mark 2014). The inclusion of Mg·ATP was associated with a highly stable structure of P-gp and confirmed the asymmetry of NBD organisation. The model was subsequently used with electron paramagnetic resonance spectroscopy

to demonstrate alternating access of TM6/12 during the translocation process (van Wonderen et al. 2014). Recently, molecular dynamic simulations have been used with the model to propose sites for binding and translocation of morphine and nicardipine (Subramanian et al. 2015).

Refinement of the Murine Crystal Structure of P-gp

Figure 3 presents a comparison of the NBD separation in mouse (3G5U, 4KSC and 4M1 M) and *C. elegans* (4F4C) structures with the distances between the Cα atoms of the last resolved residue in the C-terminus of both NBD1 and NBD2 shown. Clearly, the separation between these catalytic sites is highly variable between the four P-gp structures and the incongruity of this value may highlight the significant conformational flexibility of the protein.

Comparison of the structure from murine and *C. elegans* P-gp revealed considerable differences, most notably possible registry shifts. Consequently, in 2014, Li et al. published a refined model (4M1M) of the murine P-gp structure (Li et al. 2014) as an update to the original structure (Aller et al. 2009). The refined structure was generated using single-wavelength anomalous dispersion (SAD) phasing of the original mouse Pgp structural data. The initial dataset was produced using multi-wavelength anomalous dispersion (MAD) phasing, which has largely been superseded by SAD (Li et al. 2014). To refine the original model, SAD phasing was conducted on a previously collected dataset free of radiation damage. Registry shift corrections were made to the TM helices forming the central cavity, intracellular helices and elbow helices, amongst other corrections. The refinements resulted in a significant increase in the percentage of residues present in the favourable Ramachandran region (95 % in the refined model compared to 56 % in the original structure). The SAD-phased map showed improvement in many criteria and revealed features of the protein that were not resolved in the original model. Finally, the figure of merit (FOM) value for the refined structure improved from a MAD score classified as unacceptable to a SAD score that is.

The refined structural model for murine P-gp identified nine aromatic residues within the TMDs that are also present in human P-gp, but not in the *C. elegans* isoform (Jin et al. 2012; Li et al. 2014). The authors speculated that evolutionary differences in residue composition are indicative of different functions and account for the significant substrate promiscuity observed in mammalian P-gp isoforms. Significant changes in the position of TM4 were observed in the refined structure, and this helix is implicated in formation of the portal for drug entry. The improved murine structure is a superior template to generate new homology models in order to investigate the molecular basis for polyspecific drug recognition. The continued travails with the structure of P-gp provide considerable evidence for stringent and rigorous assessment of data, moreover, investigators should ensure equal quantities of care and of course, patience, in the fact that structural biology is a long journey.

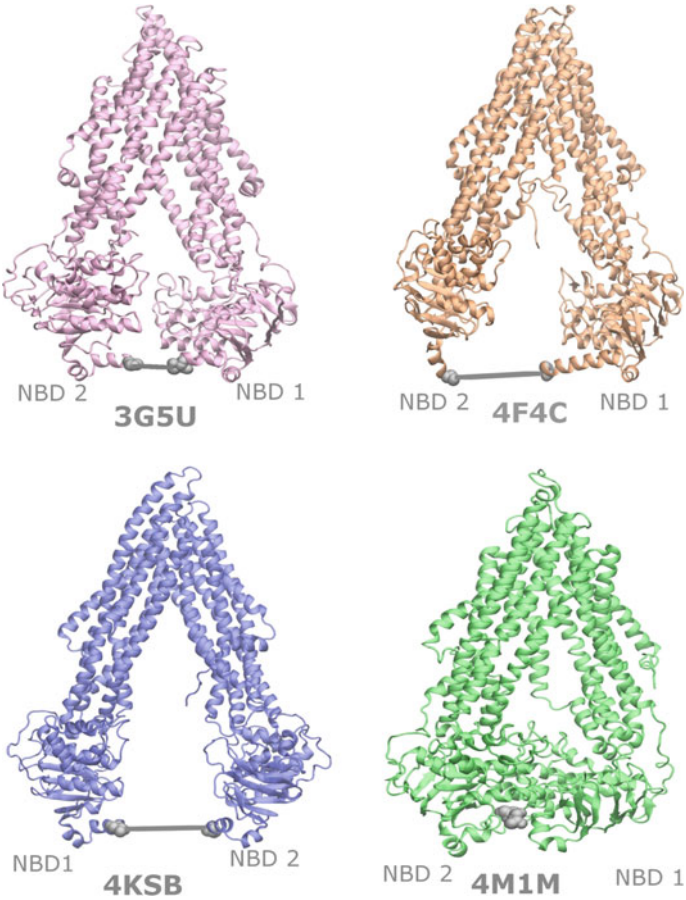

Fig. 3 Separation of nucleotide-binding domains for mouse (3G5U, 4KSB and 4M1M) and *C. elegans* (4F4C) P-gp crystal structures. The distance between the final residue in the C-terminus of each NBD was measured and denoted by *grey residues* connected by a *grey line* (where visible). For mouse P-gp structures the separation was measured between residues 626 and 1271, while the distance of separation for *C. elegans* was measured between residues 659 and 1250. Mouse P-gp 3G5U (*purple*) shows a separation of 14.90 Å while other mouse structures 4KSB (*blue*) and 4M1M (*green*) show a separation of 31.06 and 16.56 Å, respectively. The distance measured between the NBDs in the *C. elegans* structure, 4F4C (*orange*) was 34.27 Å

Portals and Drug Accessibility to the Central Cavity

Structural data has consistently shown the presence of portal(s) in the TMD formed by pairs of TM helices. The topography of these pairs is such that substrates present in the lipid bilayer can enter the binding pocket through the interface between the two TMDs. Extensive biochemical data has suggested gates are formed by the interaction of one TM helix in one TMD with another TM helix in the second

TMD. The X-ray structures of mouse P-gp have shown the presence of two portals formed by TMs 4 and 6 in TMD1 and TMs 10 and 12 in TMD2 (Aller et al. 2009; Ward et al. 2013). Only one portal capable of acting as a drug entry site was observed in the *C. elegans* structure, with the other portal sterically hindered by an N-terminal helical hairpin (Jin et al. 2012).

Do these portals merely provide an open entry point to the central cavity for drugs? Are the portals simply sites of interactions for modulators to regulate the translocation process? If the portals represent an initial binding site, then why do pharmacological studies on drug binding display a single association step? Structural investigations with P-gp have revealed information regarding the location of binding sites, the presence of a central cavity, and the identification of putative substrate portals. However, many fundamental questions regarding the drug-binding site remains, and several new questions posed in this paragraph have emerged. Only through an iterative process that combines biochemical, structural and computational studies will we finally reveal this enigmatic process.

Describing the Nature of Drug-Binding Sites

In addition to identifying their location, the nature and biophysical properties of the drug-binding sites have been studied extensively. Describing the nature of the drug–protein interactions will also shed light on the pharmacophoric features essential for high affinity binding, and reveal differences between substrates and modulators. Such essential fundamental information may also translate into improved design of anticancer drugs to avoid the clutches of P-gp, or inhibitors to halt its effect on chemotherapy efficacy. Finally, understanding how the binding sites alter their interaction with drug in multiple conformations of the protein will facilitate a molecular description of multidrug recognition and translocation.

Substrate Recognition

From the earliest investigations, the ability of P-gp to interact with an astonishing number of compounds was a “standout feature”. P-gp is one member of the triad of multidrug efflux pumps that prevent cytotoxic drug accumulation in cancer and regulate pharmacokinetic profiles in healthy individuals. However, the three pumps have distinct substrate profiles, albeit with some inevitable overlap. Clearly, there must be chemical–physical features of a drug that render it as a substrate or modulator of P-gp; in other words, the elusive pharmacophore. The most widely observed features are the hydrophobicity of substrates, a planar aromatic ring system and a cationic nitrogen moiety. A number of investigations have attempted to generate a detailed list of pharmacophoric features (Stouch and Gudmundsson 2002; Montanari and Ecker 2015; Ueda et al. 1997; Demel et al. 2008; Orlowski

and Garrigos 1999); although to date, a widely accepted and definitive set is unavailable.

Hydrogen bond formation has oft-been considered a staple requirement for high affinity binding and to maintain selective drug interaction with enzymes, receptors and transporters. With this established dogma, Anna Seelig analysed a large number of compounds known to interact with P-gp and characterised the number and location of functional groups capable of participating in hydrogen bonding (Seelig 1998). The author described three chemical units based on a fixed spatial separation of electron donor groups, and possessing any one of these features would classify a molecule as a P-gp substrate. The three chemical units were categorised as possessing a spatial separation of: (1) 4.6 ± 0.6 Å between two electron donor groups, (2) 2.5 ± 0.3 Å between two electron donor groups, or, (3) 4.6 ± 0.6 Å between three electron donor groups. Binding strength (i.e. affinity) of the compound, positively increased with the number of hydrogen donor features it exhibited. A review of structure—activity relationships (SAR) (Stouch and Gudmundsson 2002) concluded that the reliance on hydrophobicity may be an over-simplification of the drug–P-gp interaction and may simply reflect the degree of membrane intercalation. Of course, this property is a key element of the hydrophobic vacuum cleaner models of P-gp (Homolya et al. 1993; Raviv et al. 1990), which demonstrate that drugs are "extracted" from the lipid milieu. This review also suggested that the examination of hydrogen bond donor/acceptor moieties on substrates gave the most successful determinants of binding. Moreover, they stated that the presence of multiple binding sites would cloud the issue and experimental discrimination between them was an essential step; unfortunately, this has not yet been possible to achieve experimentally. Another article (Raub, 2005) also indicated that the experimental tools were not yet available to discriminate between sites and that considerably greater sophistication in SAR investigations was required. In particular, strategies to improve our understanding of the structural determinants for P-gp binding/transport will require "*...reducing molecular size, replacing electronegative atoms, blocking or masking H-bond donors with N-alkylation or bulky flanking groups, introducing constrained conformation, or by promoting intramolecular hydrogen bonds...*". These are indeed important investigative strategies. However, the current pessimism by funding councils and the pharmaceutical industry towards generating P-gp inhibitors may render this goal unattainable.

Due to the vast number of structurally different compounds P-gp can recognise, it is widely accepted that the protein–drug interactions are intricate and complex. A general pharmacophore model of P-gp drugs was hypothesised from a genetic algorithm similarity program (GASP) and relates to the verapamil-binding site. GASP analysis was used to examine a highly diverse set of compounds from a number of drug classifications, to derive a general pharmacophore for P-gp substrates (Pajeva and Wiese 2002). Multiple pharmacophore elements were proposed including: two hydrophobic regions, three hydrogen bond acceptors and one hydrogen bond donor. Interaction points for individual drugs were analysed and compared with the wide variations in specific interactions attributed to different binding modes for the compounds within the defined verapamil site. Furthermore,

the authors found the binding affinity of P-gp substrates and inhibitors was influenced by the number of pharmacophore points simultaneously involved in the interaction. The verapamil-binding site contains multiple points for hydrophobic and hydrogen bond interactions, and different drugs interact with a specific subset of these points.

Another study that focussed on understanding how P-gp recognises such chemically and structurally distinct compounds, utilised a combination of biochemical (primarily ATPase assays with multiple drugs) and molecular modelling techniques (Garrigues et al. 2002). The investigation revealed that, for the compounds tested, there were two regions for drug interaction on P-gp. On one site, drugs including cyclosporin A, verapamil, actinomycin D and peptides would compete for binding. The alternate site was exclusively for vinblastine; although it must be remembered that drugs not tested in this investigation may interact at this site. This study also concluded that the binding region of P-gp contained multiple individual sites located near to each other. Moreover, the binding affinity of drugs will depend on the distribution of recognition elements such as hydrogen bond donors and hydrophobic moieties in the site. The presence of more than one site capable of drug recognition in the overall binding region provided P-gp with its characteristic promiscuity.

Another investigation, prior to the publication of the X-ray structure of murine P-gp, generated a molecular model to examine drug binding (Vandevuer et al. 2006). The model was developed using data from a wide range of sources including electron microscopy, cysteine-disulphide cross-linking and mutagenesis data. Molecular docking programs were used to assess the binding of selected drugs, with energetics of the interaction used to assign relative potencies. Seven drugs were docked to the cavity within the centre of the protein and the nature of their interactions with the polypeptide was analysed. A number of different types of interactions between drug and P-gp were identified, which included hydrogen bonding, π–π stacking, and cation–π interactions. The findings were general support for the pharmacophore model described by Pajeva and Wiese (Pajeva and Wiese 2002). The ligands were found to bind at different positions within the cavity and multiple binding locations were observed for a single ligand. Taken together, this study provided evidence of multiple drug-binding sites and information describing the drug–protein interactions at these locations.

Do Modulators and Substrates Bind at Distinct Sites on P-gp?

The descriptions presented thus far indicate that P-gp is likely to have a large binding domain, which is likely to reside within the central cavity. A number of helices from the TMDs line the cavity and it has been proposed that substrates may enter this cavity directly from the lipid milieu through defined portals. A number of compounds have been identified that inhibit the transport of anticancer drugs by P-gp and thereby restore the efficacy of chemotherapy. Often these “inhibitors” are

classified as competitive, despite the lack of formal data to indicate binding at the identical site to substrate. In addition, many "inhibitors" of the transport of anticancer drugs are themselves substrates for transport. A classical inhibitor would prevent the transport of a true substrate, but not be translocated across the membrane per se. The interaction of two compounds that are both transported would be revealed as an apparent inhibitory effect on the compound whose transport was being measured. As will be shown in subsequent sections, there is a vast amount of data to indicate that P-gp allosteric binding sites can influence binding and transport of drugs. This intricate and complex network of interactions necessitates clarification of nomenclature. The term modulator will be used to indicate a compound that interferes with the transport process for a substrate. Moreover, the modulator must bind to P-gp at a site distinct from the substrate in question; i.e. at an allosteric site.

Photo-affinity labelling approaches demonstrated distinct binding sites for the P-gp substrate [^{125}I]-IAAP and the modulator (±) *cis*(Z)flupentixol (Dey et al. 1997, 1999; Maki et al. 2003). [^{125}I]-IAAP was shown to photo-label P-gp at two sites on the protein and that the presence of *cis*(Z)flupentixol was able to enhance the binding. The enhancement of photo-labelling (and presumably binding) is clear evidence for a positive allosteric interaction. In addition, *cis*(Z)flupentixol displayed a differential ability to alter the photo-labelling of [^{125}I]-IAAP at the N- and C-terminal sites. Also, ATP-bound and vanadate-trapped P-gp displays considerably reduced photo-labelling by [^{125}I]-IAAP and the high affinity binding site is only "regenerated" following dissociation of nucleotide (Maki and Dey 2006). However, *cis*(Z)flupentixol binding to P-gp in the ATP or vanadate-trapped configuration (without nucleotide dissociation) is also able to "regenerate" [^{125}I]-IAAP binding, suggestive of an allosteric site that is essentially unaffected by events at the NBDs (Maki and Dey 2006). *cis*(Z) flupentixol treatment of P-gp generated proteolytic cleavage patterns that were identical to those obtained following trapping of the protein with ADP-vanadate or ATP-γ-S (Ghosh et al. 2006). It appears that either binding site for *cis*(Z)flupentixol may be located in TM12, since mutation of the phenylalanine residue to alanine (F983A) affects the tryptic digestion pattern observed in the presence of modulator. Alternatively, the residue may be involved in the conformational changes accompanying *cis*(Z)flupentixol binding. The data using this strategy clearly demonstrates alternate binding sites for a modulator and substrate; in addition, binding at the two sites confers distinct conformational transitions in P-gp.

One of the X-ray structures of mouse P-gp (Aller et al. 2009) was obtained in the presence of two enantiomeric modulators that showed inhibitors bound within the central cavity. This has led to efforts aimed at localising the H- and R-sites. One investigation focussed on the ability of the two enantiomers (QZ59-RRR and QZ59-SSS) to modulate substrate transport from the H-site (Hoechst 33342) and the R-site (Daunorubicin) (Martinez et al. 2014). The SSS-enantiomer was found to alter transport of both the H- and R-site substrates in a competitive manner. The RRR-enantiomer also interacts with transport of Hoechst 33342, but with a lower potency than the SSS-enantiomer. In addition, the RRR-enantiomer inhibits

daunorubicin transport in a non-competitive manner. The data may demonstrate that the QZ59-SSS binding overlaps with both the H-site and the R-site. In addition, the binding of QZ59-RRR may occur at the H-site, but may allosterically affect the R-site. It is worth noting that the competitive and non-competitive interactions were measured exclusively with transport, and not binding assays. Binding is the first step of transport and the unequivocal attribution of the location of QZ59-SSS or QZ59-RRR binding requires alternative assay systems. Nonetheless, the data reveal that modulators and transported substrates can bind at distinct sites on P-gp; however, some sites may be competent for both transport and allosteric modulation.

Complex Interactions Between Substrates and Modulators on P-gp

The data summarised in Sect. "Do Modulators and Substrates Bind at Distinct Sites on P-gp?" (and elsewhere) clearly demonstrate that the nature and location of drug binding to P-gp is a complex one and involves an intricate network of sites on the protein. This was further illustrated by an investigation of the effects of 34 P-gp interacting drugs (i.e. substrates and modulators) on the characteristics of ATP hydrolysis (Litman et al. 1997). They then examined the interactions between these drugs by studying them in pairs; for example, using verapamil and a series of transported substrates. This ambitious study led to the identification of at least four major types of drug interactions on, or with, P-gp. These four types comprise classical competitive and non-competitive inhibition, allosteric inhibition and co-operative stimulation of ATP hydrolysis.

Another series of manuscripts also examined the effects of pairs of compounds on the ATPase activity of P-gp in order to deduce the nature of drug binding (Orlowski and Garrigos 1999; Pascaud et al. 1998; Garrigos et al. 1997). For example, Pascaud et al. measured the alteration of P-gp ATPase activity in the presence of vinblastine, azidopine, verapamil and five dihydropyridine modulators; nicardipine, nimodipine, nitrendipine and nifedipine (Pascaud et al. 1998). ATPase activity was stimulated by verapamil and nicardipine but not by vinblastine. Amongst the dihydropyridines, the potency to stimulate activity was greatest for the more hydrophobic compounds. The authors painstakingly measured and assessed the nature of interaction between the drugs. They generated a "*sticky paper model*" for the drug binding to P-gp based on the ATPase assay effects. The binding "domain" consisted of a series of individual sites for drugs; one class of site was specific for transported substrates, and others for modulators. The modulator sites affected ATPase activity of a substrate independently, while others worked in tandem. Some sites were exclusive to a class of compounds, whilst others displayed broad specificity to a number of drugs.

Collectively, the enzymatic assay results described in this section were in agreement with the concept of a multiple-site binding model for drugs. Moreover,

the sites are involved in a complex allosteric network of either positive or negative heterotropic interactions. Interpretation of the data is complex due to the use of a functional assay. ATPase activity consists of drug/nucleotide association, instigation of conformational change, ATP catalysis, nucleotide/drug dissociation and resetting. Drug interactions may affect one or more of these steps in the process.

Classical Receptor-Drug Analysis of Multidrug Binding to P-gp

As alluded to in the previous section, it is difficult to assign binding sites for drugs based on assays that involve multistep functional activity. Direct binding of drugs to proteins can be measured using radioligand-binding assays. Simple equilibrium assays provide affinities of ligands (K_D), binding capacity (B_{MAX}), the inhibition constant for modulators (K_I) and the nature of interaction between two drugs readily demonstrated by the relative effects on K_D/B_{MAX} using classic Schild plots (Larazeno and Birdsall 1993; Kenakin 1997). The use of kinetic assays to determine the association and dissociation rate constants will provide definitive proof of allosteric interactions as defined by the law-of-mass action.

Several teams used the radioligand approach to examine the binding of drugs to P-gp, both to discover novel "inhibitors" of the protein, and to reveal information on the binding interaction (Ferry et al. 1992, 1995; Martin et al. 1997). The dissociation constant (K_D) was measured for several substrates of P-gp and revealed considerable differences in the affinities of ligands to bind to P-gp. The presence of distinct binding affinities argues against one of the assumptions of the hydrophobic vacuum cleaner model, which suggested that interaction is purely based on non-specific adsorption to a hydrophobic interface. Assays measuring the heterologous displacement of radiolabelled substrates were used to estimate the potency (IC_{50}) of displacing compound to bind to P-gp. However, the IC_{50} value is only a relative measure of affinity that may be converted to an inhibition constant (i.e. K_I) using the Cheng–Prusoff equation (Larazeno and Birdsall 1993). This transformation is only applicable in cases where the displacing compound and radioligand share a competitive interaction. Consequently, the dissociation rate constant was also measured for the radioligand and the ability of displacing compound to affect this rate was determined. Several dihydropyridines, Hoechst 33342 and Tariquidar (to name a few), were able to increase the rate of [^{3}H]-vinblastine dissociation from P-gp (Ferry et al. 1995; Martin et al. 1997; Pascaud et al. 1998). According to the law-of-mass action, this can only occur through an allosteric effect; thereby, confirming the presence of multiple drug-binding sites.

Another approach to discriminate between competitive and non-competitive interaction involves the use of Langmuir Adsorption (Saturation) Isotherms to analyse radioligand-binding assays (Kenakin 2004). Saturation isotherms were generated for [^{3}H]-vinblastine in the presence of increasing concentrations of other

substrates or modulators (Martin et al. 2000). An alteration in the B_{MAX} for [^{3}H]-vinblastine was observed in the presence of the substrates Rhodamine123, Hoechst 33342 and the modulators XR9051, XR9576 (Tariquidar) and GF120918 (Elacridar). The reduced B_{MAX}, in the absence of any alteration in the apparent K_D for [^{3}H]-vinblastine, was indicative of a non-competitive interaction. The non-competitive interaction was confirmed as a negative allosteric heterotropic one following demonstration of an elevated dissociation rate constant (k_{off}). Based on the data with [^{3}H]-vinblastine, it appears that other transported substrates bind at distinct sites on P-gp. Similarly, the modulators also interact at a site distinct from that of [^{3}H]-vinblastine. Is there any overlap, or does the binding site on P-gp contain a distinct interaction "zone" for all compounds?

To address this question, similar analyses were undertaken using a tritiated version of the most potent P-gp modulator, XR9576 (i.e. Tariquidar) (Martin et al. 2000). In the presence of vinblastine, the B_{MAX} for [^{3}H]-XR9576 was unaffected, however there was a dose-dependent shift to the right in its saturation binding curves. The data was analysed using a Schild plot, which was characterised by a slope significantly different to 1.0, which is indicative of a non-competitive interaction. This is in full agreement with data obtained for [^{3}H]-vinblastine in the presence of XR9576. Hoechst 33342 also produced a shift in the saturation binding curves of [^{3}H]-XR9576 to the right. However, in the case of Hoechst 33342, the slope was not significantly different to 1.0 and revealed a competitive binding interaction with [^{3}H]-XR9576. Whole cell accumulation assays indicated that [^{3}H]-XR9576 was not a substrate for transport by P-gp and the unlabelled compound also inhibited the ATPase activity of purified protein (Martin et al. 1999). The binding data revealed that XR9576 and the transported substrate, vinblastine, bound at distinct sites on P-gp. However, since XR9576 interacts with P-gp at the same location as the transported substrate Hoechst 33342, their shared site is indeed capable of transport. Why is [^{3}H]-XR9576 not transported by P-gp? The rate of [^{3}H]-XR9576 dissociation from P-gp was considerably slower than that observed for vinblastine or paclitaxel. The high affinity binding of [^{3}H]-XR9576, in particular the slow k_{off} rate, may prevent efficient transport across the bilayer since rapid dissociation from the external facing binding site is essential for transport. This suggests that it is the nature of the ligand and its interaction with the binding site that determine whether transport across the bilayer occurs.

The data in the preceding paragraph demonstrate that the modulator XR9576 and the substrate Hoechst 33342 bind at the same site. Is this a generic property of the binding sites; namely that they are capable of interacting with either class of compounds? This would confirm that the nature of the ligand, rather than the site properties, dictates whether transport occurs. To address this, the interaction of a number of other modulators with [^{3}H]-XR9576 was investigated.

In particular, the effects of nicardipine, XR9051 and GF120918 (Elacridar) on the saturation isotherms and the dissociation rate of [^{3}H]-XR9576 were measured. Both nicardipine and XR9051 increased the apparent K_D of [^{3}H]-XR9576 without any effect on the B_{MAX}, which is suggestive of competition. XR9051 did not alter the dissociation rate of [^{3}H]-XR9576, thereby confirming a competitive interaction;

which is not unexpected given the structural similarity of the two ligands. In contrast, nicardipine caused an increase in the dissociation rate, indicating a non-competitive allosteric interaction. Consequently, nicardipine must bind at a site distinct from the binding site shared by XR9576, XR9051 and Hoechst 33342. GF120918 caused a dose-dependent decrease in the B_{MAX} of [^{3}H]-XR9576 and [^{3}H]-vinblastine without a change in the K_D, indicating that GF120918 does not bind to the same sites as either. Another study had demonstrated a non-competitive interaction between GF120918 and, which suggests the presence of a fourth site on P-gp.

Collectively, the evidence collected from these investigations was used to classify at least four distinct drug-binding sites on P-gp. This provided crucial support not only for the existence of multiple binding sites on P-gp but also insight into the complex ways in which the binding sites communicate with each other. The data also demonstrated that substrates and modulators can bind at equivalent sites. It is therefore the nature of interaction at the site that demonstrates whether a drug will be transported.

Summary and Perspectives

Over more than three decades, scientists have attempted to localise drug-binding sites and to provide detail on the molecular interactions with P-gp. These attempts have involved biochemistry, pharmacology, structural and bioinformatics approaches. The purpose of this article was to summarise the numerous sources of data for this key question in the ABC transporter field. We have moved on considerably since the early *hydrophobic vacuum cleaner* and *drug-flippase* models. However, drugs do appear to enter the binding domain directly from the lipid milieu and do so via portals or gates formed at the interface between the two TMDs. The binding domain is located in the central cavity of P-gp, which is lined by multiple helices. The precise dimensions of this cavity remain the source of considerable debate within the structural biology field. The binding domain contains multiple sites for interaction with drugs and the drug–protein interactions involve a combination of hydrogen bonding and π–π stacking, particularly with phenylalanine residues. Initial binding of a drug causes local conformational changes leading to a substrate-induced fit that generates high affinity. The individual sites are proximal and each appears capable of interacting with transported substrates and modulators. Moreover, it is the molecular interaction between drug and site that determines whether translocation of drug across the membrane occurs.

The primary objective remains to unequivocally determine the residues involved in the interaction with drugs at the individual sites within the domain.

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The ABCG2 Multidrug Transporter

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Abstract ABCG2 is an ATP-binding cassette (ABC) half-transporter localized to the cell membrane and important in normal physiology, in normal tissue protection including in the maternal-fetal barrier and the blood brain barrier, and potentially in multidrug resistance. Dimerization is required for function. Polymorphic variants have been described that impair trafficking to the cell surface, and one, Q141K, has been associated with a higher incidence of gout in individuals who are carriers. Working in concert with ABCB1 (P-glycoprotein), ABCG2 is expressed at high levels in the vascular endothelium of the brain and pumps xenobiotics and chemotherapeutics back into the bloodstream. ABCG2 expression is also found at high levels in some cancer types including pancreatic and liver cancers. Whether its expression in these tumor types contributes to drug resistance via drug efflux and reduction in drug accumulation is a question that remains unanswered. Studies of drug accumulation, such as through imaging solid tumors in patients, are needed to answer this question.

Introduction to ABCG2

The human ATP-binding cassette (ABC) transporters are transmembrane efflux transporters that belong to a large superfamily of 48 members including ABCG2, or human breast cancer resistance protein (BCRP), encoded by the *ABCG2* gene located on chromosome 4q22.

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ABCG2 is a 655 amino acid, 72 kDa protein consisting of an N-terminal nucleotide-binding domain (NBD) containing an ABC signature motif (ALSGGQ) and a C-terminal transmembrane domain (TMD) with six hydrophobic segments and an extracellular loop between TM5 and TM6. Its structure is in reverse configuration to most other ABC transporters containing one or two NBDs that are C-terminal to the TMDs. ABCG2 is a half-transporter (one NBD and one TMD), requiring at least two NBDs to function as an efflux pump; thus, functional ABCG2 exists as either homodimers or homomultimers (Bhatia et al. 2005; Doyle and Ross 2003; Kage et al. 2002; Nakanishi et al. 2003; Xu et al. 2004). Homodimer formation involves a disulfide bridge linkage at cysteine 603 located in the extracellular loop (Henriksen et al. 2005; Wakabayashi et al. 2007). Crystallization studies reveal a tetrameric complex comprised of four homodimers (McDevitt et al. 2006) and other studies have shown that it can also form higher oligomeric states (Xie et al. 2008; Xu et al. 2007). It is speculated that ABCG2 dimerizes in the endoplasmic reticulum (ER), exits to the Golgi apparatus for posttranslational processing, and is finally then trafficked to the apical membrane of the cell (Graf et al. 2003).

This chapter is about ABCG2—its expression in normal tissues, substrate/inhibitor specificity, role in cancer multidrug resistance, and particularly its role in the blood–brain barrier (BBB). Understanding how genetic variations affect transporter function or substrate and inhibitor specificity and drug–transporter interactions at the BBB will shed light on improving drug delivery to the brain. The contribution of ABCG2 to multidrug resistance in the tumor vasculature versus the BBB will be addressed followed by a brief discussion on prospects for transporter inhibition at the BBB.

Tissue Expression

Since ABCG2 was first discovered in drug-resistant human cancer cells, subsequent studies focused on its pattern of expression in various normal tissues, where ABCG2 was found to be involved in the absorption, distribution, metabolism, and elimination of xenobiotics and endogenous chemicals. The location and level of expression of ABCG2 highlighted major roles for ABCG2 in forming the maternal–fetal barrier, the blood–testis cell barrier, and the blood–brain barrier; controlling the absorption and efflux of xenobiotics and endogenous metabolic products within the gastrointestinal tract; and more recently its role in urate transport in the kidney.

Placenta and Mammary Gland

The highest levels of ABCG2 expression are found in placental tissue, specifically in the placental syncytiotrophoblast at the apical surface of the chorionic villi. Due to this specific localization, it is thought that ABCG2 helps form the barrier between

the maternal and fetal circulation systems, and thus protects the fetus from endogenous and exogenous toxins (Fetsch et al. 2006). It is also possible that ABCG2 transports steroid hormones produced in the placenta (Doyle and Ross 2003), since the major estrogens produced and secreted by the placenta are ABCG2 substrates (Suzuki et al. 2003).

While ABCG2 is expressed at low levels in the non-lactating adult breast, its expression is elevated in lactating mammary tissue, localized in the lobules and lactiferous ducts of the mammary gland, as well as in venous/capillary endothelium (Maliepaard et al. 2001; Faneyte et al. 2002). As opposed to the protective role ABCG2 may play in the placenta, the functional role of ABCG2 here would be to concentrate substrates such as vitamins—as has been demonstrated for riboflavin—or other essential factors into breast milk; however, enrichment of toxins in breast milk has also been demonstrated (Jonker et al. 2005; van Herwaarden et al. 2006, 2007).

Testis

High expression of ABCG2 has been reported in normal testis tissue, where it is expressed in the Sertoli–Leydig cells, in the myoid cell layer, as well as in endothelial cells (Fetsch et al. 2006; Bart et al. 2004). Due to its localization on the luminal side of the endothelium as well as the apical side of myoid cells, it probably transports substrates out of the seminiferous tubules, implying a potential role in protecting the germ cells.

Blood–Brain Barrier

ABCG2 was found to be expressed on the luminal surface of brain microvessel endothelium, suggesting a role in the blood–brain barrier (Cooray et al. 2002). Since then, numerous researchers have explored the role of ABCG2 in forming the blood–brain barrier (BBB), predominantly in murine models. ABCG2 was found to be expressed along with P-gp (ABCB1) in the microvasculature of both normal and malignant tissues from the central nervous system, but their relative functional contribution still needs to be determined. Not only do studies indicate that ABCG2 plays a role in the efflux of toxic xenobiotics in the BBB, but it also appears that it could play a role in efflux of endogenous substrates, since its activity in the transport of efflux of amyloid-β peptide was recently reported (Do et al. 2012; Tai et al. 2009).

Liver and the Gastrointestinal Tract

When Taipalensuu et al. (2001) examined jejunal mucosa from 13 normal volunteers, and compared expression levels of 10 drug efflux transporters from the ABC

family, the highest level detected was for the mRNA encoding ABCG2. Other studies indicated that ABCG2 mRNA was detected at high levels in the duodenum, decreasing along the gastrointestinal tract, and reaching lowest levels in the rectum (Gutmann et al. 2005; Hilgendorf et al. 2007). ABCG2 was localized by immunohistochemistry at the apical membrane of the small intestine, the colon, and the liver bile canaliculi. A recent quantification of intestinal ABCG2 from 14 human donors indicates that the average BCRP density in the human duodenum is 305 ± 248 fmol/cm2 and it was found at 2.6 pmol per gram in the liver (Tucker et al. 2012).

The presence of ABCG2 in the apical membrane of the small intestine and bile canniculi implies a role for the protein in xenobiotic and endogenous substrate efflux from the liver and substrate absorption in the GI tract (Adachi et al. 2005). Another role for ABCG2 in the intestine is in the secretion of endogenous uric acid, even though renal excretion is the most important factor in uric acid elimination (Hosomi et al. 2012).

Kidneys

BCRP is expressed at the apical membrane of proximal tubules, suggesting a role for BCRP in renal excretion of xenobiotics (Fetsch et al. 2006; Huls et al. 2008). In 2008, a genome-wide association study (GWAS) reported that a common single nucleotide polymorphism of ABCG2 was linked to elevated blood urate level and gout (Dehghan et al. 2008). Further studies revealed that ABCG2 was indeed involved in excretion of urate in urine, again highlighting the importance of ABCG2 in the transport of endogenous compounds (Woodward et al. 2009).

Other Sites

BCRP is expressed in a wide variety of stem cells including those of hematopoietic origin and is the molecular determinant of the side population (SP) phenotype (Zhou et al. 2001; Scharenberg et al. 2002). SP cells have been identified by their ability to effectively exclude the fluorescent vital dye Hoechst 33342 and form a distinct population, definable by flow cytometry (Goodell et al. 1996).

ABCG2 mRNA expression was reported in multiple other tissues (see Fig. 1), and various studies confirmed ABCG2 protein expression in alveolar pneumocytes, sebaceous glands, transitional epithelium of the bladder, prostate epithelium, uterine endocervical cells, cervical squamous epithelia, small and large intestinal mucosa/epithelial cells, pancreatic islet and acinar cells, zona reticularis layer of the adrenal gland, gall bladder, conjunctival epithelium, retinal pigment epithelium, spinal cord, and hepatocytes (Fetsch et al. 2006; Maliepaard et al. 2001; Aust et al. 2004; Chen et al. 2013).

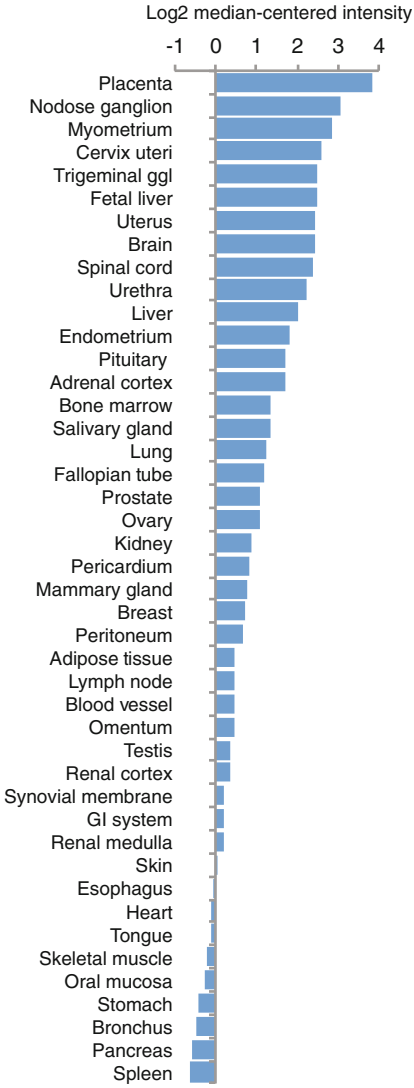

Fig. 1 Log2 median-centered ABCG2 expression data obtained from analysis of 502 normal tissues, source GEO accession number GSE7307 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE7307). Selected samples were grouped by organ and median expression per organ was calculated

Polymorphisms and ABCG2 Null Humans

Structural and functional studies reveal that ABCG2 transporter activity is affected by both posttranslational modifications and genetic polymorphisms. During its transit through the endoplasmic reticulum–Golgi pathway to the plasma membrane, ABCG2 undergoes N-linked glycosylation at asparagine 596 (Diop and Hrycyna 2005) and formation of an intramolecular disulfide bond between C592 and C608, where mutating these cysteine residues results in impaired localization and function (Henriksen et al. 2005; Wakabayashi et al. 2007). The oligomeric glycosylated ABCG2 undergoes lysosomal degradation whereas the underglycosylated, misfolded protein lacking the intramolecular disulfide bond is degraded via the proteasome (Wakabayashi et al. 2007; Nakagawa et al. 2009; Wakabayashi-Nakao et al. 2009). Certain non-synonymous single nucleotide polymorphisms (SNPs), such as Q141 K, F208S, and S441 N, affect ABCG2 protein stability resulting in enhanced ubiquitin-mediated proteasomal degradation (Furukawa et al. 2009; Nakagawa et al. 2008). Moreover, Q141 K and F208S variants are found sequestered in the aggresome followed by subsequent degradation via autophagy (Basseville et al. 2012; Wakabayashi-Nakao et al. 2010). In addition, mutational studies of ABCG2 identified a GXXXG motif (residues 406–410), responsible for dimerization, where mutation of the glycines to leucines affected the protein's function (Polgar et al. 2004). Mutational analysis of threonine 402, three residues from the GXXXG motif, reveals a role for this residue in modulating protein folding and processing as well as dimerization (Polgar et al. 2010). Furthermore, functional variations in *ABCG2* have been shown to affect substrate binding and specificity. Mutating Arg482 and Pro485, located in the drug-binding pocket within TM3, can affect the efflux of some substrates (Ni et al. 2011).

More than 80 single nucleotide polymorphisms in the *ABCG2* gene have been identified across ethnically diverse subpopulations. Among them, Q141K (421C > A, rs2231142) and V12M (34G > A, rs2231137) occur most frequently in Asians (~25–35 %) and with lower frequencies in Caucasians (~10 %), and African-American populations (~<5 %), while the remaining SNPs have allelic frequencies of less than 2 % (Backstrom et al. 2003; de Jong et al. 2004; Iida et al. 2002; Imai et al. 2002; Kobayashi et al. 2005; Kondo et al. 2004; Zamber et al. 2003). Functional studies revealed that the Q141K and D620N mutations decrease ABCG2 activity, while V12M, I206L, and N590Y do not appear to alter protein function (Morisaki et al. 2005; Vethanayagam et al. 2005). Other non-synonymous SNPs, F208S (623T > C, rs1061018) and S441 N (1321G > A), lead to reduced protein stability by enhancing proteasomal proteolysis (Furukawa et al. 2009). In fact, S441N displayed lower protein expression and impaired membrane localization (Kondo et al. 2004). Moreover, an in vitro study found 6 of the 18 ABCG2 variants to exhibit defective or impaired transport of the substrates hematoporphyrin or methotrexate: Q126stop (376C > T, rs72552713); F208S; S248P (742T > C, rs3116448); E334stop (1000G > T, rs3201997); S441 N; and F489L (1465T > C) (Tamura et al. 2006). Additional SNPs reported in the *ABCG2* promoter region,

−15622C > T and −1379A > G, may be involved in transcriptional control of the gene (Noguchi et al. 2014). The nonsense mutation Q126X (376C > T, rs72552713) introduces a stop codon resulting in a non-functional transporter (Matsuo et al. 2009).

Q141K and Drug Disposition/Clinical Outcome

Pharmacogenetics studies of ABCG2 have mainly focused on the Q141K variant. Q141K is associated with reduced protein expression (Imai et al. 2002; Morisaki et al. 2005) possibly due to increased ubiquitin-mediated proteasomal degradation (Furukawa et al. 2009), as well as reduced protein function. The SNP is located in the ATP-binding region between the Walker A and B motifs of ABCG2 and has also been associated with reduced ATPase activity, impaired membrane localization, and instability in the nucleotide-binding domain (Imai et al. 2002; Kondo et al. 2004; Mizuarai et al. 2004; Woodward et al. 2013). These findings indicate that this SNP may result in altered transporter function and have important implications for regulating drug disposition. However, studies evaluating the clinical relevance of this SNP have been inconsistent largely due to the small sample size of the study populations. Patients with the variant genotype (AA or CA) had significantly higher plasma AUC or Cmax levels for statins (Keskitalo et al. 2009a, b; Lee et al. 2013; Zhang et al. 2006; Zhou et al. 2013), TKIs (sunitinib Mizuno et al. 2012 and imatinib Takahashi et al. 2010), sulfasalazine (Urquhart et al. 2008; Yamasaki et al. 2008) as well as the camptothecin derivatives diflomotecan (Sparreboom et al. 2004) and 9-aminocamptothecin (Zamboni et al. 2006). However, no significant effects of the Q141 K variant on pharmacokinetic parameters were observed for irinotecan (de Jong et al. 2004; Han et al. 2007; Jada et al. 2007), docetaxel (Chew et al. 2011), or nitrofurantoin (Adkison et al. 2008). Furthermore, this SNP has also been linked with increased drug-induced diarrhea for Korean patients with diffuse large B-cell lymphoma receiving rituximab plus cyclophosphamide/doxorubicin/vincristine/prednisone (Kim et al. 2008) or patients on gefitinib therapy (Cusatis et al. 2006). The 421AA genotype correlated with risk of grade 3 or grade 4 thrombocytopenia and neutropenia in Korean patients with metastatic renal cell carcinoma on sunitinib therapy (Kim et al. 2013).

The association of Q141 K with clinical outcome has also been mixed with a study demonstrating longer progression free survival for the variant allele in advanced ovarian cancer patients (Tian et al. 2012) and other studies showing poor outcome for patients with acute myeloid leukemia being treated with idarubicin-based therapy (Tiribelli et al. 2013) or lung cancer patients on a platinum-based regimen (Muller et al. 2009).

The other common ABCG2 SNP, 34G > A, has not been reported to significantly affect drug pharmacokinetics. The *ABCG2* (−15622C/T) polymorphism and the *ABCG2* (1143C/T, −15622C/T) haplotype were associated with gefitinib-induced moderate-to-severe diarrhea, but their functional impact is still unknown (Lemos

et al. 2011). Other genetic polymorphisms have been identified in the coding region of ABCG2 but their clinical relevance remains to be determined (Noguchi et al. 2014; Zhao et al. 2009).

ABCG2 Null Alleles

ABCG2 was identified as the genetic basis of a blood group system named Junior, Jr or Jr(a), such that null alleles of *ABCG2* cause the Jr(a−) blood type. Individuals who do not express Jr(a) antigen are rare and are mainly found in Japanese and other Asian populations. SNP analysis on genomic DNA from 6 Jr(a−) subjects led to the identification of three nonsense mutations (Q126X, Q246X, and R236X) where all three SNPs resulted in a premature stop codon in the ATP-binding domain of ABCG2 (Zelinski et al. 2012). A separate study also sequenced *ABCG2* in 18 Jr (a−) individuals and found eight mutations: three nonsense mutations described above (Q126X, R236X, and Q246X) and five frameshift mutations (I63Yfs, F182Vfs, L264Hfs, F293Lfs, and T371Lfs) (Saison et al. 2012). Individuals were either homozygous for a single *ABCG2* mutation or heterozygous for two mutations. Both studies concluded that the *ABCG2* mutations identified in the Jr(a−) individuals correspond to null alleles of *ABCG2* and are responsible for the Jr(a−) blood type. Ongoing studies have continued to identify additional new null alleles in Jr(a−) patients (Hue-Roye et al. 2013a, b; Ogasawara et al. 2014; Tanaka et al. 2014) and the functional significance is still unknown.

Substrates and Inhibitors of ABCG2

Substrates

The ABCG2 substrate spectrum has increased exponentially over the years to include physiological compounds, cancer and non-cancer therapeutics, molecularly targeted drugs, and common dietary xenobiotics. ABCG2 is involved in the physiologic efflux of multiple endogenous substrates that include conjugated organic anions (Mao and Unadkat 2015; Polgar et al. 2008); porphyrin/heme (Jonker et al. 2002; Krishnamurthy et al. 2004); folates (Chen et al. 2003); and urate (Woodward et al. 2009) as well as amyloid-β peptides across the BBB (Xiong et al. 2009).

Since it was originally discovered in drug-resistant cells, the first compounds reported as (exogenous) substrates of ABCG2 were chemotherapeutics that include mitoxantrone (Doyle et al. 1998), anthracyclines, methotrexate, camptothecin derivatives, and flavopiridol as well as tyrosine kinase inhibitors (TKIs) such as imatinib and gefitinib (Mao and Unadkat 2015; Polgar et al. 2008). Drug-selected

cell lines that express mutant forms of ABCG2 (R482G and R482T) were found to modify substrate selectivity resulting in enhanced efflux of rhodamine 123 and increased resistance to anthracyclines (Chen et al. 2003; Allen et al. 2002a; Honjo et al. 2001). Subsequent site-directed mutagenesis studies at amino acid 482 further demonstrated its importance in determining substrate recognition and transport (Miwa et al. 2003; Ozvegy-Laczka et al. 2005; Robey et al. 2003). However, it should be noted that mutations of Arg482 have never been identified in clinical samples to date.

Other substrate classes have been identified and include certain antibiotics (fluoroquinolones and erythromycin), antiretrovirals (nucleoside analogs, zidovudine and abacavir), carcinogens, flavonoids (genistein and quercetin), and HMG-CoA reductase inhibitors (Mao and Unadkat 2015; Polgar et al. 2008). Fluorescent probes have been reported to be ABCG2 substrates and include Hoechst 33342, BODIPY-prazosin, LysoTracker Green, and pheophorbide A (Litman et al. 2000; Robey et al. 2004). The transport of photosensitizers by ABCG2 further implicates this transporter as a possible cause of cellular resistance to photodynamic therapy (Robey et al. 2005). More recently, D-luciferin was identified as a specific ABCG2 substrate that can be used as a probe for (bioluminescent) imaging of ABCG2 function at the BBB in vivo (Bakhsheshian et al. 2013a).

ABCG2 has a broad substrate specificity that is distinct, but substantially overlaps, with that of P-gp and multidrug resistance protein (MRP1). Although the number of ABCG2 substrates exceeds 200, few studies have been conducted to determine structure–activity relationship (SAR) for prediction of ABCG2 substrates. One SAR study conducted with camptothecin analogs showed that ABCG2 transports compounds with high polarity at carbon positions 10 and 11 (Yoshikawa et al. 2004). A recent study by Hazai et al. (2013) developed a predictive model for wild-type ABCG2 substrates, using a support vector machine (SVM) method based on 263 known BCRP substrates and non-substrates, with an overall prediction accuracy of $\sim$76 % for identifying potential novel substrates.

Inhibitors

The first ABCG2 specific inhibitor identified was the diketopiperazine fumitremorgin C (FTC) isolated from the fungi *Aspergillus fumigatus* (Rabindran et al. 1998). FTC was reported to inhibit mitoxantrone resistance in drug-selected cell lines even before ABCG2 was cloned, but had no effect on resistance mediated by P-gp or MRP1 (Rabindran et al. 1998). Because of its neurotoxic effects, FTC analogs (Kol43) were soon developed with more potent inhibitory activity and high selectivity for ABCG2 and low neurotoxicity (Allen et al. 2002b; van Loevezijn et al. 2001).

Many ABCG2 inhibitors are not selective and can inhibit other ABC transporters, such as the potent dual P-gp/ABCG2 inhibitors elacridar (de Bruin et al. 1999) and tariquidar (Robey et al. 2004) as well as the growing list of TKIs

(Durmus et al. 2015). Additional classes of inhibitors include flavonoids, steroids, nucleoside analogs, and immunosuppressants (tacrolimus, cyclosporine A). Chromone derivatives and tariquidar-like structures are considered the most potent and selective inhibitors of ABCG2, inducing high inhibition of ABCG2 with low effect on ABCC1 and ABCB1 (Lecerf-Schmidt et al. 2013). Given the promiscuous nature of ABCG2, the list of inhibitors (and substrates) will continue to expand in the coming years.

The mechanism of inhibition divides ABCG2 inhibitors into two groups: "general" inhibitors that inhibit ATPase activity of the transporter such as FTC and Ko143; and "substrate-dependent" inhibitors that are ABCG2 substrates and thus act as competitive inhibitors. The latter group results in substrate-dependent inhibition where the inhibitor may interact with ABCG2 on (i) binding sites of one class of substrates but not others, or (ii) on allosteric sites to induce conformational changes in the large binding pocket that subsequently affects transport of certain substrates. For example, nelfinavir has been shown to inhibit the efflux of zidovudine and abacavir with no effect on efflux of prazosin and imatinib, suggesting that the nucleoside analogs possibly bind to ABCG2 at sites that do not overlap with those for prazosin or imatinib (Giri et al. 2009). These findings support the notion that substrates interact with ABCG2 at multiple binding regions in the protein. For this reason, predicting and screening for ABCG2 inhibitors remains a challenge in drug discovery.

Role of ABCG2 in Cancer Multidrug Resistance

The story of ABCG2 in multidrug resistance is inextricably linked with that of P-gp/ABCB1. Discovered in 1976 in drug-resistant Chinese hamster ovary cells, the therapeutic potential was quickly recognized when verapamil was found to overcome resistance by blocking drug efflux (Tsuruo et al. 1981; Juliano and Ling 1976). Other inhibitors were rapidly discovered and the idea was translated to the clinic with the first trial result reported in 1987, a trial combining verapamil with adriamycin in ovarian cancer (Ozols et al. 1987) relying on data showing that high levels of resistance due to P-gp-mediated efflux could be overcome by addition of P-gp inhibitors. For over a decade, many publications began with some variation of 'drug resistance is a major problem in the treatment of cancer', and then went on to report the detection of P-gp expression in a new tumor type or the identification of a new inhibitor to overcome resistance. Eventually it became apparent that there were drug-resistant cell lines with high levels of drug efflux but in which P-gp expression was not detectable. MRP was discovered as a mediator of drug efflux in some of those cell lines (Schneider et al. 1994), and then ABCG2 (Ross et al. 1999). However, because the discovery of P-gp preceded those of MRP and ABCG2 by two decades, it was translated to the clinic on its own.

From the first clinical trial in 1987, a series of studies testing a variety of P-gp substrates and inhibitors in patients with a variety of tumor types was carried out.

Although many trials were conducted with off-the-shelf agents developed for another purpose, a number of later P-gp inhibitors were developed specifically for that purpose. In sum, a handful of trials had a statistically significant positive outcome, but most of the studies were unconvincing and did not support a role for overcoming resistance by adding a P-gp inhibitor. In retrospect, there were mistakes in the development that may have obscured a positive signal. These included a delay in the conduct of randomized trials, failure to selectively enroll only patients whose tumors had detectable P-gp expression prior to enrollment, and utilization of inhibitors that also altered the pharmacokinetics of the anticancer drug in the combination such that lower drug doses had to be used (Amiri-Kordestani et al. 2012; Shaffer et al. 2012). A large number of trials were conducted with inconclusive results. Two randomized trials were launched in 2002—with tariquidar, a very potent P-gp inhibitor, combined with vinorelbine or paclitaxel plus carboplatin in non-small cell lung cancer. The trials were conducted at about 100 centers around the U.S., and were closed within a year for toxicity in the experimental arm, with only 304 of an expected 1000 patients enrolled (Fox and Bates 2007). The most positive signal had come from an acute leukemia study with cyclosporine A in 2001, with multiple intervening studies inconclusive (Shaffer et al. 2012). A third generation P-gp inhibitor, zosuquidar, was conclusively negative (Cripe et al. 2010). In none of the trials were patients selected because their tumors demonstrated expression of P-gp—and in the ensuing years we have learned just how important it is that the target be demonstrated in the tumor before expecting any activity from a targeted agent (Bates et al. 2012). In these studies, P-gp was the obvious target.

In this climate of disappointment, ABCG2 and ABCC1 were discovered and came of age as potential targets for cancer therapy. It was not long before inhibitors of both were discovered and with that the need to identify which tumors were ABCG2-expressing and which were P-gp-expressing. Given that the P-gp clinical trials were turning up negative, and that a clinically validated assay for P-gp had never been developed, the road to an ABCG2 inhibitor in the clinic seemed very long indeed. VX-710, biricodar, was an P-gp and ABCC1 modulator that was thought to also inhibit ABCG2 (Minderman et al. 2004). But a single arm Phase II trial of VX-710, doxorubicin, and vincristine did not meet a level of interest to be pursued (Gandhi et al. 2007). Nor were studies in ovarian, breast, or prostate cancer (Seiden et al. 2002; Toppmeyer et al. 2002; Bramwell et al. 2002; Rago et al. 2003). Notably all these studies enrolled patients with refractory or relapsed cancers and none selected patients based on transporter expression. VX-710 also required dose reduction for the anticancer agent (Toppmeyer et al. 2002; Rago et al. 2003). This is as close to an ABCG2 inhibitor trial as the field came. The disappointment with P-gp inhibitors led to a halt of almost all such clinical development, although the FDA and industry continued to be interested in the role of ABCG2 and its polymorphic variants in the disposition of substrate drugs (Mao and Unadkat 2015).

Furthermore, this impasse diverted interest in drug transporters to their role in normal tissue protection, including a focus on their role in the blood–brain barrier.

Perhaps time and improving technology will allow a reassessment of the role of drug transporters in cancer drug resistance. The sequencing methodologies employed by The Cancer Genome Atlas (TCGA) also yield gene expression quantitation. The greater sensitivity of this method has allowed detection of *ABCG2* and *ABCB1* in a large number of clinical cancer samples. As shown in Fig. 2a, the range of *ABCG2* expression is over 1000-fold among eight different tumor types shown in the dot plot: breast, pancreas, kidney, liver cancers, and glioblastomas and sarcomas. The range of expression is striking and suggests that for some tumor types the transporter expression may be higher and potentially more important than recently considered. Further, the co-expression with *ABCB1* is very striking in these tumor types, with $r = 0.659$. In Fig. 2b, colorectal cancer samples have been added. Here the correlation has fallen to 0.479 because the colon cancer samples express *ABCB1* more than *ABCG2*. In Fig. 2c, data for glioblastoma and low-grade gliomas are shown. Here a tight correlation between *ABCG2* and *ABCB1* is shown. Interestingly, relative to other tumor types, the level of expression in the glioblastomas is high, with low-grade glioma higher still, perhaps linked to normal tissue expression.

This striking analysis raises several questions. One is whether inhibition of one transporter could ever be enough in tumors that express both pumps (and see the data for murine BBB knockout models below). A second is whether the correlative studies reporting poor outcome with expression of only one transporter may have been reflecting the totality of transporter presence. Note that the question of whether drug transporters impact drug accumulation in tumor tissue has never been satisfactorily answered; studies measuring drug in tumor tissue are generally old and generally show wide interpatient variability. Clinical imaging tools are critically needed to address the question. Asking whether an P-gp/ABCG2 inhibitor could improve outcome was not the right question. Rather, the question that needs to be addressed is whether the presence of drug transporters reduces drug accumulation in tumor tissue. Certainly data shown below, gathered in murine models for normal brain penetration, imply that the P-gp/ABCG2 combination could have a tremendous impact.

The Blood–Brain Barrier

Introduction to the Blood–Brain Barrier

The blood–brain barrier (BBB) is a dynamic network of cells and proteins that regulates the molecular communication between the blood and most of the central nervous system (CNS) (Pardridge 2012). The brain and spinal cord actively adjust

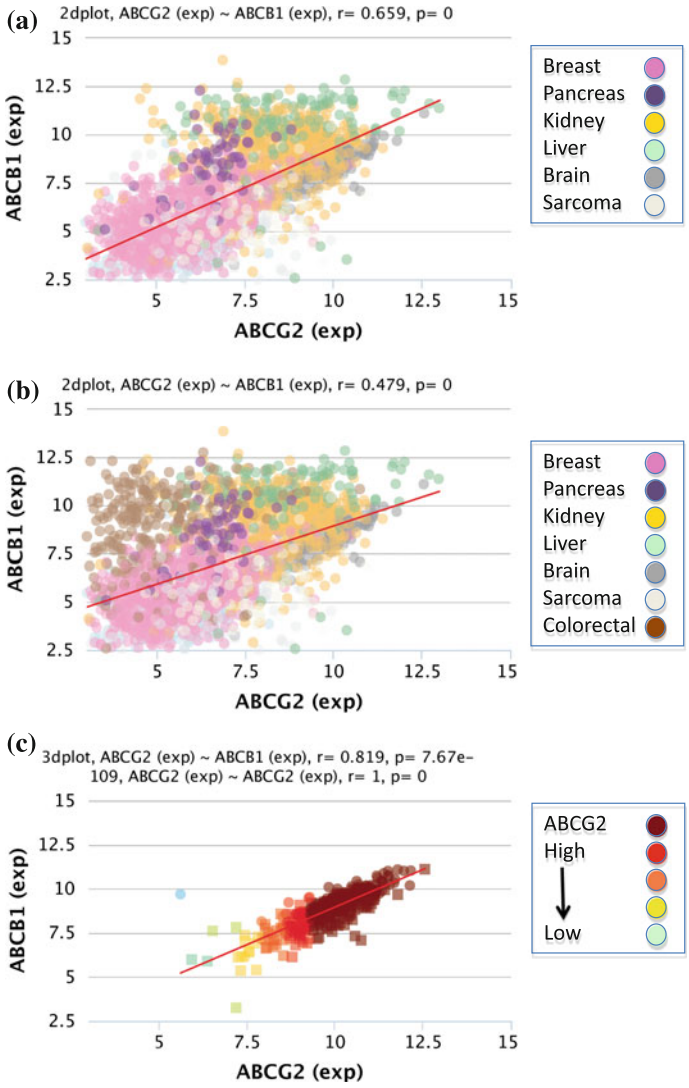

Fig. 2 Expression data for ABCB1 and ABCG2 in tumor samples in the TCGA dataset. **a** Tumor sample expression of *ABCB2* and *ABCB1* in the TCGA dataset is shown, with tissue of origin color-coded. Number of samples from TCGA depicted: breast cancer = 994; pancreatic cancer = 56; kidney cancer = 480; hepatocellular cancer = 134; glioblastoma = 169; sarcoma = 77. **b** Colorectal samples (n = 233) shown in addition to the samples depicted in (**a**). **c** Expression data for glioblastoma (n = 169, *squares*) and low-grade glioma (n = 275, *circles*) are shown; *darker color* represents higher transport expression with highest levels found in low-grade glioma. Data for all three graphs were acquired from CellMiner, courtesy of Rajapakse (2015)

to daily variations of nutritional and oxygen requirements while maintaining the capacity to respond to pathologic alterations in the microenvironment such as increased intracranial pressure and toxic materials in the systemic circulation (Saunders et al. 2014). The BBB presents clinical challenges in the context of vasogenic edema, neuro-imaging, and drug delivery for various pathologic conditions including tumors, epilepsy, and infection (Löscher and Potschka 2005).

A small molecule can traverse a biological barrier in several ways: by passing through the capillary cells by either passive diffusion or facilitated transport (transcellular), by passing between the endothelial cells (paracellular), or by being taken up by the cell (pinocytosis) and across the cell (transcytosis) (Pardridge 2012; Abbott 2013). The BBB protects the brain by regulating and restricting each of the described pathways. Firstly, brain capillary cells form a physical barrier preventing paracellular transport. This is achieved by capillary endothelial cells forming tight junctions via proteins such as the connexins and claudins, and are characterized by the visualization of 'kissing points' via electron microscopy (Bauer et al. 2014). An underlying basement membrane and astrocytic end-feet have also been postulated to provide an additional barrier to permeability, and the maintenance of the BBB architecture has been shown to be regulated by astrocytes and pericytes that are immediately basolateral to the basement membrane (Cabezas et al. 2014; Muoio et al. 2014). Furthermore, BBB sites have fewer pores between the endothelial cells and decreased pinocytic activity compared to peripheral endothelial cells (Georgieva et al. 2014). Therefore, in a normal physiological setting, drug delivery to the brain parenchyma relies primarily on transcytosis, and this is consistent with the observation that for many organic small molecules, brain penetrance positively correlates with the partition coefficient (logP) for those molecules (Levin 1980). However, there are outliers to this relationship. The classic example is D-glucose, whose uptake to the brain is much greater than anticipated based on logP, due to the expression of high-affinity glucose uptake transporters (such as GLUT1) at the apical surface of brain endothelial cells, and carrier-mediated transport is responsible for the uptake of amino acids and other molecules essential for metabolic function (Cornford and Hyman 2005).

The other critical role for the BBB is that of active defense, precluding entry into the brain of unwanted small molecules that are capable of passive diffusion or 'hijacking' passive transporters. This is achieved by the expression of ATP-Binding Cassette (ABC) transporters at the apical surface, primarily the drug transporters ABCG2 and ABCB1 (see next section) (Kannan et al. 2009). Here, transporters can intercept small molecules at the apical cell membrane (often referred to as the 'lipid-membrane vacuum cleaner' model), and efflux them back into the blood plasma against the concentration gradient (Gottesman et al. 2002). Drug metabolizing cytochromes are also expressed in brain endothelial cells, and ABCC1 and ABCC4 are also expressed at the apical surface—these have been associated with the transport of secondary metabolites, such as glutathione conjugates (Cole 2014). These protective processes are highly energy (ATP) dependent, and this is one of the reasons that brain endothelial cells are mitochondria-rich (Hicks et al. 1983). We present a summary of transporters expressed at the BBB in Fig. 3.

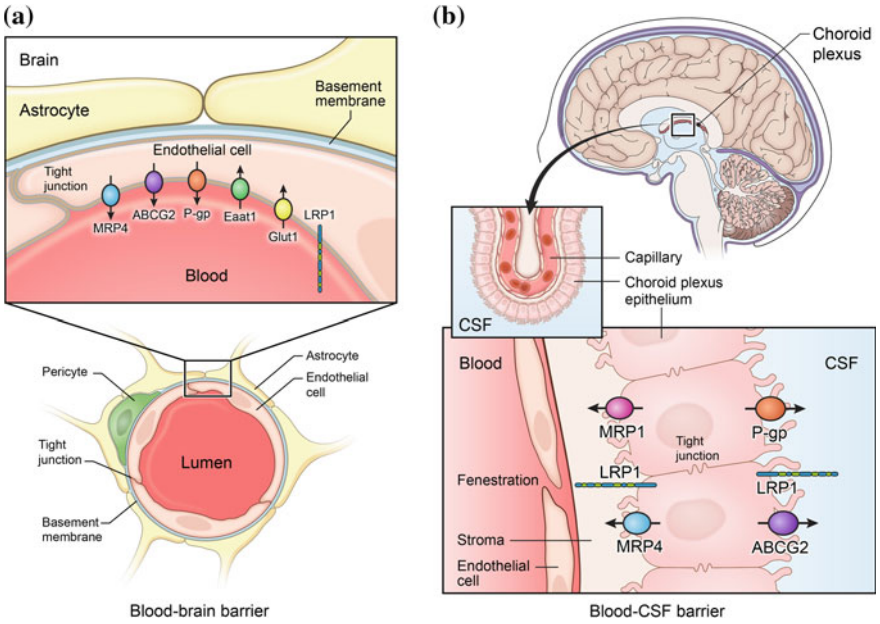

Fig. 3 Schematic representation of **a** the blood-brain barrier and **b** the blood-CSF barrier. The blood-brain barrier (**a**) is formed primarily by brain endothelial cells in capillaries, and is regulated by surrounding pericytes and astrocytes at the basolateral side of the endothelial cells. The endothelial cells form tight junctions, mediated by connexin, occludin and claudin family proteins. At the apical cell surface, ABC transporters such as P-gp (P-glycoprotein, *ABCB1*), ABCG2 (also breast cancer resistance protein) and MRP4 (multidrug resistance protein 4, *ABCC4*) transport small molecules back into the lumen. Ingress of nutrients from the blood supply is mediated by facilitative solute carrier SLC transporters, such as glutamate (excitatory amino acid transporter 1, Eaat1, *SLC1A3*) and D-glucose (glucose uptake transporter 1, Glut1, *SLC2A1*). LRP1 (low-density lipoprotein receptor-related protein 1 receptor, APOER) mediates receptor-mediated transcytosis across brain endothelial cells, and is one of several receptor targets present on the apical side of the BBB being evaluated for brain-targeted therapeutics. Lining the apical surface and projecting into the lumen is the glycocalyx (not shown), composed of glycoprotein and polysaccharide. The blood-CSF barrier (**b**) is distinct from the BBB and exists as part of the choroid plexus, responsible for producing CSF. It is comprised of epithelial cells forming tight junctions, while the endothelial capillary cells that supply nutrients for CSF production are fenestrated, allowing protein access to the choroid plexus epithelium. ABC transporter expression is a matter of some debate, but there appears to be general agreement that MRP (ABCC) transporters are oriented in the epithelium towards the blood, and that P-gp and ABCG2 are oriented towards the CSF. This has led to the hypothesis that the choroid plexus supplies drug to the CSF, and that this is a 'back door' for drug access to the brain parenchyma, and the follow-on conclusion that drug CSF levels are an acceptable surrogate indicator of drug brain concentrations. However, there is now clear evidence that there is a large divergence between brain and CSF levels for most drugs. Figure was prepared with the assistance of Alan Hoofring of NIH Medical Arts and Printing

ABC Transporter Expression at the BBB

Numerous studies have examined the expression of ABC transporters in brain capillary cells. It was recognized very early on that P-glycoprotein was expressed in brain endothelial cells, and ABCG2 expression was reported shortly after the gene itself was identified. The functional contribution of ABCG2 in protecting the BBB has been examined for a range of therapeutics. The most direct insights have been derived from gene knockout mice (see elsewhere in this chapter). For many agents the cross-recognition of substrates by P-gp and ABCG2 indicates a cooperativity in their protective role at the BBB. This is significant given the recognition that a population of humans of the Jr(a) blood group carry two missense copies of ABCG2 and do not express functional ABCG2 protein. Given the lack of reported drug-related adverse neurologic events in these patients, it is likely that redundancy with P-gp minimizes the impact of the loss of ABCG2.

The absolute levels of ABC transporter expression have been assessed, and the work of Terasaki and co-workers will be highlighted here. They have measured the expression of transporters in isolated brain capillary endothelial cells and in cell culture using quantitative liquid chromatography-tandem mass spectrometry (Kamiie et al. 2008). This method allows for measurement of protein levels, usually given as fmol/μg of cellular protein in endothelial cells isolated from brain capillaries of species including mice, monkeys, and humans. Primary findings regarding expression will be conveyed here, along with specific observations related to ABCG2. Assessment is carried out by first isolating brain capillaries from brains of the species of interest, exclusion of large vessels (that are unlikely to be capillaries), and subsequently isolating protein from the resulting capillaries (Ohtsuki et al. 2014).

A summary of expression of ABC transporters is shown in Table 1. It should be noted that ABCG2 is comprised of two functional protein units and as such two protein molecules form one functional transporter, so, for example, 14 fmol/μg ABCG2 protein results in 7 fmol/μg functional ABCG2 protein. Given that the functional capacity of ABC transporters is so high, the functional consequences could be concluded to be irrelevant (the so-called 'spare transporter' effect) (Kalvass and Pollack 2007). In mouse brain capillaries, P-gp (mdr1a, 12–15 fmol/μg protein) expression was higher than Abcg2 (4–5 fmol/μg protein), and MRP4 (ABCC4) was also detectable (Kamiie et al. 2008). The second mouse P-gp, Mdr1b, was not detectable. As a means of comparison, the highest transporter protein expression was for Glut1 (90 fmol/μg protein). Expression in adult and younger cynomolgus monkeys revealed differences from mouse, the primary difference being that ABCG2 expression (14 fmol/μg protein) was higher than P-gp (5–6 fmol/μg protein) (Ito et al. 2011). There was no difference in ABCG2 expression between adult and infant monkeys, though P-gp expression was slightly higher in infants. In Sprague-Dawley and Wistar rats, levels of P-gp (Abcb1, 19 fmol/μg protein) and Abcg2 (2–3 fmol/μg protein) were approximately equivalent between the two breeds, and Abcc4 (MRP4, 1–2 fmol/μg protein) was also detectable (Hoshi et al. 2013). In porcine brain

Table 1 Reported expression levels (fmol/μg) for various ABC efflux transporter proteins in various species

	ABCG2	*ABCB1*	*ABCC4*	*ABCA2*	*ABCA8*
	Bcrp	P-gp	MRP4		
Human (healthy)	4.1	6.1	0.2	2.9	1.2
Human (diseased)	4.0	3.1	0.3	2.1	0.7
Monkey	7.1	4.7	0.3	NR	NR
Marmoset	8.3	6.5	0.3	NR	NR
Sprague-Dawley rat	2.1	19.0	1.6	NR	NR
Wistar rat	2.9	19.2	1.5	NR	NR
Mouse	2.2	14.1	1.6	ULQ	ULQ
hCMEC/D3 cells	1.1	3.9	0.3	7.2	ULQ

ABCG2/Bcrp values are half that reported, to account for the homodimerization of protein product to form a functional unit
ULQ Under limit of quantification; *NR* Protein not reported/examined

capillaries, BCRP was more highly expressed than P-gp, but comparison with other species is complicated by an alternative analysis technique (Kubo et al. 2015). The expression of fractionated lysate was assessed to determine the localization of transporters toward the luminal (apical) or abluminal (basolateral) fraction (Kubo et al. 2015). P-gp and ABCG2 were almost exclusively located in the luminal fraction, but this does not indicate all transporters are at the cell surface, and a significant portion may be located in recycling endosomal compartments below the apical cell surface. Curiously, the cholesterol transporter ABCA1 was also detectable, but its expression was toward the abluminal surface of capillary cells. It is worth noting that in the above studies multiple other ABC transporters were assessed for expression and were not detectable, such as ABCB5, MRP1-3, ABCG5, and ABCG8 (Ito et al. 2011).

Two studies examining absolute transport protein levels in human brain microvessels have been reported; the first utilizing capillaries from brains of male patients who died of peripheral disease (Uchida et al. 2011), the second from patients with brain pathologies such as epilepsy or glioma (Shawahna et al. 2011). Major challenges in working with human brain samples are the time from death till autopsy, the tissue harvesting protocols employed, and freezer storage time, which are known to generally impact protein expression levels (Hynd et al. 2003; Harrison et al. 1995). In brains without a brain pathology, ABCG2 was more highly expressed (3–11 fmol/μg protein but with less functional protein than P-gp as explained above) than P-gp (4–9 fmol/μg protein) and MRP4 (0.1–0.3 fmol/μg protein). Of note, the lysosomal transporter ABCA2 and the proposed lipid transporter ABCA8 were also detectable (Uchida et al. 2011). Capillaries derived from brain pathologies did not deviate significantly from those of a healthy brain (Shawahna et al. 2011). An informative comparison was made between human brain microvessels and the human brain capillary endothelial cell line hCMEC/D3 (Ohtsuki et al. 2013). Strikingly, P-gp was undetectable in cells, and ABCG2

expression (1–2 fmol/μg protein) was reduced compared with fresh brain microvessels. Furthermore, ABCA2 expression (11 fmol/μg protein) was greater than brain microvessels (2–3 fmol/μg protein), and overall the correlation between primary tissue and cell culture was poor.

A controversial area of study has been that of the expression of ABC transporters in astrocytes, and whether expression occurs in the end-feet processes and is part of the BBB transport capacity. Early immunohistochemical studies of human brain reported that P-gp localized to cells also expressing glial fibrillary acidic protein (GFAP), a marker for astrocytes, but not with the brain endothelial cell marker GLUT1. This led to the hypothesis that transporters are found predominantly at the astrocyte foot processes and not in the endothelial luminal membrane (Golden and Pardridge 2000). Multiple issues arose from this model, that required drug to cross endothelial cells into the brain extracellular fluid before being pushed back across endothelial cells into the vasculature. The observations were critically discussed in the literature (Schinkel 1999), and multiple follow-up studies have demonstrated that high expression of P-gp occurs on the apical surface of capillary cells, with little to no P-gp expression in astrocytes (Loscher and Potschka 2005). Several pathologies associated with neuroinflammation, such as drug-resistant epilepsy, have led to increases in expression of P-gp in astrocytes, but this is not generally proposed to contribute to the barrier function of the neurovascular unit (Loscher and Potschka 2005).

Drug–Transporter Interactions at the BBB

Understanding the role of ABCB1 and ABCG2 at the blood–brain barrier has been aided by the study of brain penetration of various substrate drugs in murine knockout experiments. First, studies were performed in mice in which Abcb1a and Abcb1b had been deleted—the human ABCB1 equivalent. Further exploration demonstrated that when Abcg2 was deleted the effects on drug accumulation in the CNS were modest, but that when both were deleted there could be markedly greater brain penetration of substrates. Recently a study went one further step, deleting Abcc4 as well and evaluating topotecan brain accumulation (Lin et al. 2013). This was important because ABCC4 is a multidrug transporter also expressed at the blood–brain barrier. The studies demonstrated the additive effect of ABCC4 together with P-gp and ABCG2 in restricting brain penetration. The heat map in Fig. 4 depicts the results with the double knockout, *Abcb1a/b* and *Abcg2*, and shows how neither knockout alone has a major impact on the accumulation of most substrates, *but that together the impact is clinically important and statistically significant for most substrates*.

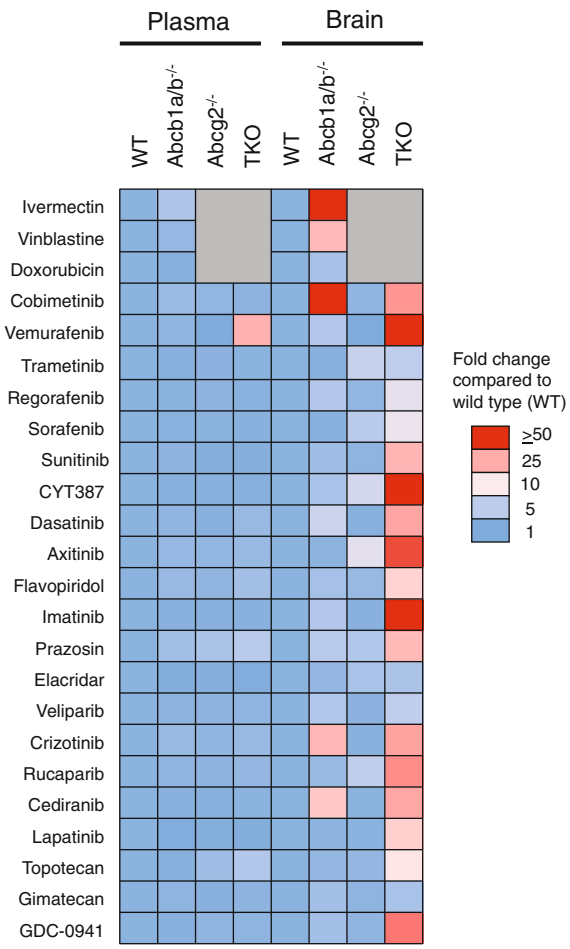

Fig. 4 Role of ABCG2 and ABCB1 in plasma or brain concentrations of chemotherapy drugs. Values shown are fold increase compared to wild-type mice, regardless of the parameter examined. Inspection of the heat map indicates that transporter knockout (in the murine model) seldom affects pharmacokinetics, and single transporter knockout seldom affects brain penetration. But double knockout has a marked effect on the brain accumulation of substrates. Data abstracted and calculated from references—using absolute concentrations where possible, to calculate fold change over wild-type (Lin et al. 2013; Schinkel et al. 1994; van Asperen et al. 1999; Choo et al. 2014; Mittapalli et al. 2012; Vaidhyanathan et al. 2014; Kort et al. 2015; Lagas et al. 2010; Oberoi et al. 2013; Durmus et al. 2013; Chen et al. 2009; Poller et al. 2011; Zhou et al. 2009; Sane et al. 2013; Lin et al. 2014; Chuan Tang et al. 2014; Durmus et al. 2015; Wang et al. 2012; Polli et al. 2009; de Vries et al. 2007; Salphati et al. 2010)

Brain Metastases and Tumor Vasculature

One area still in need of investigation is that of expression of ABCG2 and P-gp in brain tumors and brain metastases. The blood–brain barrier becomes partially disrupted in patients with both glioblastomas and brain metastases from different solid tumors. This is readily demonstrated by the MRI visualization of gadolinium contrast agent, which represents the leakage of gadolinium into the tumor. This leakage has caused some investigators to conclude there is no blood–brain barrier left in individuals with these malignancies. However, a careful observation shows that certain areas of tumors are enhanced with contrast while others are not; just as certain drugs work better than others in treating malignancies involving the CNS. The best evidence suggests that some components of the BBB remain (endothelial cells, astrocytes, basal lamina, pericytes, etc.) and contribute to the multidrug resistance seen in malignant disease in the CNS. This evidence has been gathered by studying drug concentrations in metastatic lesions in the brain in preclinical models, and reinforced by clinical studies. In brain metastases derived from MDA-MB-231 cells in nude mice, a range of paclitaxel concentrations was detected, from 22 ng/g—approximating normal brain at 14 ng/g—to 1400 ng/g, and everything in between (Lockman et al. 2010). Only 15 % of tumors had levels at the low end approximating normal brain, while 10 % of tumors had levels at the highest end, still far below the 10,000 range measured in systemic metastases. These data convincingly show that the vestiges of the BBB remain in intracranial metastases, and explain why the occasional patient shows a marked and unexpected response with an agent not thought to cross the blood–brain barrier. A recent clinical study suggested a similar range of drug penetration in metastases from patients (Morikawa et al. 2015). Levels of lapatinib varied from 1.0 to 6.5 μM and capecitabine from 0.12 to 1.97 μM. Together, these data suggest that the blood–brain barrier is leaky and aberrant in individuals with brain tumors, and the regulation of the ABC transporters in that setting not really known.

Prospects for Transporter Inhibition at the BBB

The question then becomes whether we could improve cancer therapy in the CNS by combining therapy with an efflux inhibitor. Certainly an efflux inhibitor would be most valuable when the blood–brain barrier is still intact—at a time when preventing the early development of CNS metastases would be most likely to succeed. Such a prevention strategy has been successfully explored in animal models (Palmieri et al. 2014).

Drug delivery to the CNS represents a major challenge for chemotherapeutics due to the existence of efflux transporters such as P-gp and ABCG2 at the BBB that affect drug distribution. Studies have shown that these two transporters function in concert, or even synergistically (Zhou et al. 2009). A rational approach is the

deliberate modulation of these transporters to mediate the permeability of substrates across the BBB (the "modulation of efflux" approach). However, it has also been suggested that clinical inhibition of P-gp and ABCG2 function at the BBB may not be pharmacologically achievable (Kalvass et al. 2013).

Development of approaches to successfully modulate ABCG2 activity at the BBB depends largely on improving and validating reliable BBB models for extrapolation of rodent data to humans. First, the overlapping substrate and inhibitor specificity of human and mouse ABCG2 supports the use of mouse models to evaluate the clinical, physiological, and pharmacological roles of ABCG2, especially at the BBB (Bakhsheshian et al. 2013b). Second, ABCG2 levels are reported to be similar (less than twofold difference) between murine and human BBB (Uchida et al. 2011). Third, any model must take into account the overlapping substrate specificity of ABCG2 and ABCB1 and ABCC4, all at the BBB. A recent study identified D-luciferin as a specific probe for bioluminescence imaging of ABCG2 function in vivo at the BBB (Bakhsheshian et al. 2013a). This novel method will allow for better understanding of the kinetics of transporter activity at the BBB. The study further demonstrated that ABCG2 plays a role in limiting the biodistribution of a specific substrate, and the possibility for saturable transporter inhibition at the BBB using pharmacologic levels of inhibitor, which was not possible before for ABCG2. Such tools will aid drug discovery studies with novel modulators of the transporter and/or new methods to increase drug delivery to the brain.

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Mechanistic and Pharmacological Insights into Modulation of ABC Drug Transporters by Tyrosine Kinase Inhibitors

Suneet Shukla, Atish Patel and Suresh V. Ambudkar

Abstract ATP-binding cassette (ABC) drug transporters have both physiological and pharmacological importance in humans. They not only protect the cells from xenobiotics, but also alter the pharmacokinetics and toxicity of drugs that are substrates for these transporters. P-glycoprotein (P-gp) was the first human ABC transporter to be discovered, almost four decades ago. Drug transport using the energy harvested by ATP hydrolysis is a hallmark of ABC drug transporters and has been extensively studied to determine its mechanism of substrate specificity and transport activity. Structural information from a number of human P-gp homologs highlights the existence of multiple drug-binding sites that can interact with a diverse set of chemically or structurally unrelated compounds. Tyrosine kinase inhibitors (TKIs) comprise a class of drugs that interact with ABC transporters with high affinity and are reported to be transport substrates of several ABC transporters. A number of preclinical and clinical studies have shown that ABC transporters influence the disposition of several TKIs that include altered pharmacokinetics and safety profiles and have a role in the development of resistance to this class of therapeutics. Structural details regarding the TKIs' effect on ABC transporters have not been thoroughly explored. This review summarizes preclinical and clinical observations on the interaction of TKIs with ABC drug transporters. In addition, we provide structural information on the interaction of two TKIs, nilotinib and imatinib, at the drug-binding regions within P-gp and ABCG2. This information could help to generate a novel scaffold that would be an ideal TKI with potent inhibition of kinases, but minimal interactions with ABC drug transporters.

Keywords ABC transporters · ABCG2 · Multidrug resistance · P-glycoprotein · Pharmacokinetics · Targeted therapeutics · Tyrosine kinase inhibitors

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Abbreviations

ABCB1	ATP-binding cassette subfamily B member 1
ABCG2	ATP-binding cassette subfamily G member 2
ALK	Anaplastic lymphoma kinase
AML	Acute myeloid leukemia
BCR-ABL	Breakpoint cluster-Abelson
BCRP	Breast cancer resistance protein
CML	Chronic myeloid leukemia
EGFR	Epidermal growth factor receptor
FAK	Focal adhesion kinase
FGFR	Fibroblast growth factor receptor
GIST	Gastrointestinal stromal tumor
HER2	Human epidermal growth factor receptor 2
IAAP	Iodoarylazidoprazosin
JAK	Janus kinase
MAPK	Mitogen-activated protein kinase
MDR	Multidrug resistance
MRP1	Multidrug resistance-associated protein 1
MRP7	Multidrug resistance-associated protein 7
NSCLC	Non-small-cell lung adenocarcinoma
P-gp	P-glycoprotein
PDGFR	Platelet-derived growth factor receptor
RTK	Receptor tyrosine kinase
TMD	Transmembrane domain
TKIs	Tyrosine kinase inhibitors
VEGFR	Vascular endothelial growth factor receptor

Introduction

An anticancer drug category that has generated widespread interest in the last decade is the tyrosine kinase inhibitors (TKIs), owing to their targeted therapeutic approach. TKIs emerged as potent inhibitors of receptor tyrosine kinase (RTK) activity after intensive structural exploration by empirical screening and iterative medicinal chemistry that resulted in compounds with ATP-competitive inhibitors having Ki values in the picomolar concentration range (Fry et al. 1994; Ward et al. 1994; Osherov and Levitzki 1994). Nearly 10,000 new patents have been filed since 2001 in the United States alone (Akritopoulou-Zanze and Hajduk 2009) and more agents are being explored for their therapeutic effects against cancer, mainly due to the fact that kinases are closely involved with the growth,

proliferation, and survival of cancer cells and are also among the most frequently mutated oncogenes and tumor suppressors (Wood et al. 2007; Parsons et al. 2005). However, progress in the field of TKIs has been rather uneven; TKIs have revolutionized the treatment of a select group of diseases with multiyear survival rates such as chronic myeloid leukemia (CML) and gastrointestinal stromal tumors (GIST), both driven by a single oncogenic kinase (Druker et al. 2001, 2006; Heinrich et al. 2003). Unfortunately, treatment with TKIs has not been very successful in cancers with high mortality rates such as lung, breast, colorectal, pancreatic, and prostate cancer, for which kinase inhibitors can prolong survival by only a few months (Shepherd et al. 2005; Sandler et al. 2006; Geyer et al. 2006; Miller et al. 2007; Jonker et al. 2007; Moore et al. 2007). TKIs become ineffective when intrinsic or acquired drug resistance develops in cancer cells (Longley and Johnston 2005). One of the mechanisms responsible for the development of drug resistance is the overexpression of ATP-binding cassette (ABC) transporters, which mediate the efflux of substrate drugs of multiple structurally and mechanistically unrelated agents. Among 48 members of the ABC transporter superfamily, P-glycoprotein (P-gp, ABCB1), breast cancer resistance protein (BCRP, ABCG2), multidrug resistance protein 1 (MRP1, ABCC1), and multidrug resistance protein 7 (MRP7, ABCC10) are associated with the development of multidrug resistance (MDR) in cancer cells to various drugs including TKIs. It is also quite possible that due to its overlapping substrate specificity with ABCG2, multidrug resistance protein 4 (MRP4, ABCC4) may be modulated by TKIs. Ubiquitous expression and distribution of these transporters along with their broad substrate profile makes them key players in a physiological defense system, often altering the disposition of administered drugs by influencing their absorption, distribution, and elimination.

In this chapter, we provide a review of existing information on TKIs, specifically concerning interactions with ABC transporters and possible therapeutic implications. In addition, the analysis of structural features of two representative TKIs (imatinib and nilotinib) that are responsible for interactions with ABC transporters will be discussed, as well as the relevance of this information to building structural analogs that retain potent TKI activity with minimum interaction with ABC efflux systems. There are several excellent reviews addressing various aspects of TKIs (Anreddy et al. 2014a; Knight et al. 2010; Greuber et al. 2013; Fruman and Rommel 2014; Pao and Chmielecki 2010; Durmus et al. 2015). We focus here on the pharmacological and mechanistic interaction of TKIs with ABC transporters.

ABC Transporters and Drug Disposition

The widespread expression of P-gp in normal tissues, including the brain and testis endothelial cells, adrenal glands, the endometrium of a pregnant uterus, renal proximal tubules, biliary canalicular membranes of hepatocytes, and the apical

surface of polarized mucosal cells in the small and large intestines (Thiebaut et al. 1987; Cordon-Cardo et al. 1989), suggests its role in creating an efflux barrier system for these organ sites, thus protecting them against a broad range of xenobiotics. P-gp has the ability to mediate the vectorial efflux of literally hundreds of structurally diverse nonpolar, weakly amphipathic substrates, ranging from fluorescent and synthetic compounds to natural products. ABCG2, another transporter now gaining interest in the field of pharmacokinetics and drug distribution, is often co-expressed alongside P-gp in the brain and testis endothelial cells, the placenta, liver, kidney, and the gastrointestinal tract. Moreover, it is also expressed in breast mammillary glands, where either alone or in conjunction with P-gp, it maintains an efficient barrier efflux system (Lee et al. 2006; Maliepaard et al. 2001). In addition, expression of ABCG2 in the apical membrane of the renal tubules and bile canalicular membranes makes it an important determinant of drug disposition and elimination. P-gp and ABCG2 help protect liver hepatocytes by efflux of xenobiotics into the bile, where the high concentration of these drugs is then carried to the intestines. Epithelial cells lining the intestines are exposed to a high concentration of xenobiotics, where the concentration gradient favors their reuptake into the cells. Thus, these powerful efflux pumps are necessary to avoid toxic accumulation of drugs within the intestinal cells (Elferink and Groen 2002; Faber et al. 2003; Alpini et al. 2002; Da Silva et al. 2015), limit accumulation, and prevent crippling adverse cellular reactions. Owing to the broad substrate specificity of these ABC transporters, the U.S. Food and Drug Administration (FDA) and the European Medicines Agency mandate careful screening of drugs for potential substrate or inhibitory activity toward these transporters (Draft Guidance for Industry 2012; Guideline on the Investigation of Drug Interactions 2012).

TKIs and Their Interaction with ABC Transporters

The interaction of TKIs with ABC drug transporters has been extensively studied in recent years (Burger et al. 2004; Dai et al. 2008; Tiwari et al. 2013; Elkind et al. 2005; Erlichman et al. 2001; Shi et al. 2007; Shukla et al. 2008a; Yanase et al. 2004). The majority of these TKIs have been shown to be transport substrates of ABC drug transporters in multiple studies. Therapeutically, these interactions of TKIs with drug transporters result in altered pharmacokinetics or the development of resistance. In vitro data from several groups, including ours, complemented by in vivo studies collectively suggest that two major transporters that may influence the clinical outcome of TKI therapy are P-gp and ABCG2 (Table 1). In particular, TKIs also inhibit the transporters' function to varying degrees, often resulting in adverse drug–drug interactions. Further, bioavailability of TKIs that are substrates of these transporters could change significantly by inhibition or induction of ABC transporters or by co-administration of another drug, leading to clinically relevant

Table 1 Interaction of TKIs with ABC transporters

RTK	TKI	ABC transporter(s) affected	Disease	Experimental data							
				IAAP labeling[a]	ATPase[b]	Transport substrate[c]	In vitro[d]	Reversal of MDR[e]	Reversal conc.[f]	In vivo[g]	Plasma conc.[h]
BCR-ABL	Bosutinib	P-gp, ABCG2	CML	ND	+ (Hegedus et al. 2009)	ND	+ (Hegedus et al. 2009)	ND	ND	ND	0.14 μM (Cortes et al. 2011)
	Dasatinib	P-gp, ABCG2	CML	+ (Dohse et al. 2010)	+ (Hegedus et al. 2009; Dohse et al. 2010)	+ (Hegedus et al. 2009; Dohse et al. 2010)	+ (Hegedus et al. 2009; Dohse et al. 2010)	+ (Dohse et al. 2010)	10 μM	–	0.100 μM (Bradeen et al. 2006)
	Imatinib	P-gp, ABCG2, MRP7	CML	+ (Shukla et al. 2008a; Brendel et al. 2007)	+ (Shukla et al. 2008a; Brendel et al. 2007)	+ (Breedveld et al. 2004; Dohse et al. 2010)	+ (Houghton et al. 2004; Hegedus et al. 2009)	+ (Gao et al. 2006)	5 μM (Peng et al. 2012; Demetri et al. 2009)	+ (Mahon et al. 2003; Peng et al. 2012)	2.4 μM
	Nilotinib	P-gp, ABCG2, MRP7	CML	+ (Brendel et al. 2007; Shukla et al. 2014)	+ (Brendel et al. 2007; Shukla et al. 2014)	+ (Dohse et al. 2010)	+ (Tiwari et al. 2009; Shen et al. 2009)	+ (Tiwari et al. 2009; Shen et al. 2009)	5 μM (Tiwari et al. 2009, 2013; Deininger 2008)	+ (Tiwari et al. 2013)	1.7 μM
	Ponatinib	P-gp, ABCG2, MRP7	CML	+ (Sen et al. 2012)	+ (Sen et al. 2012)	ND	+ (Sen et al. 2012; Sun et al. 2014)	+ (Sen et al. 2012; Sun et al. 2014)	200 nM (Sen et al. 2012; Sun et al. 2014)	ND	0.040 μM (Gozgit et al. 2012)
EGFR	Afatinib	ABCG2	NSCLC	ND	+ (Wang et al. 2014b)	ND	+ (Wang et al. 2014b)	+ (Wang et al. 2014b)	1 μM (Wang et al. 2014b)	+ (Wang et al. 2014b)	0.5 μM (Wind et al. 2013)
	AG1478.	P-gp, ABCG2	Research purposes only	+ (Shi et al. 2009)	+ (Shi et al. 2009)	ND	+ (Shi et al. 2009)	+ (Shi et al. 2009)	10 μM (Shi et al. 2009)	ND	ND
	ARRY-334543	ABCG2	Metastatic breast cancer	+ (Wang et al. 2014c)	+ (Wang et al. 2014c)	ND	+ (Wang et al. 2014c)	+ (Wang et al. 2014c)	1 μM (Wang et al. 2014c)	ND	ND

(continued)

Table 1 (continued)

RTK	TKI	ABC transporter(s) affected	Disease	Experimental data							
				IAAP labeling[a]	ATPase[b]	Transport substrate[c]	In vitro[d]	Reversal of MDR[e]	Reversal conc.[f]	In vivo[g]	Plasma conc.[h]
	AST1306	P-gp, ABCG2	Advanced solid tumors	ND	ND	ND	Zhang et al. (2014a)	+ (Zhang et al. 2014)	1 μM (Zhang et al. 2014)	ND	0.223 μM (Zhang et al. 2014c)
	Canertinib	P-gp, ABCG2	NSCLC	ND	ND	+ (Minocha et al. 2012a)	+ (Erlichman et al. 2001)	ND	ND	+ (Erlichman et al. 2001)	0.36 μM (Calvo et al. 2004)
	Erlotinib	P-gp, ABCG2, MRP7	NSCLC	+ (Shi et al. 2007)	+ (Shi et al. 2007)	+ (Marchetti et al. 2008)	+ (Shi et al. 2007; Kuang et al. 2010)	+ (Shi et al. 2007; Kuang et al. 2010)	20 μM (Shi et al. 2007; Kuang et al. 2010)	+ (Marchetti et al. 2008)	3.2 μM (Ling et al. 2006)
	Gefitinib	P-gp ABCG2	NSCLC	ND	ND	+ (Lemos et al. 2009)	+ (Yanase et al. 2004; Lemos et al. 2009)	+ (Yanase et al. 2004; Lemos et al. 2009)	5 μM (Yanase et al. 2004; Lemos et al. 2009)	+ (Kitazaki et al. 2005)	0.19 μM (Swaisland et al. 2005)
	Icotinib	ABCG2	NSCLC	+ (Wang et al. 2014b)	+ (Wang et al. 2014b)	ND	+ (Wang et al. 2014b)	+ (Wang et al. 2014b)	1 μM (Wang et al. 2014b)	+ (Wang et al. 2014b)	5 μM (Liu et al. 2014)
	Lapatinib	P-gp, ABCG2, MRP7	Metastatic breast cancer	+ (Dai et al. 2008)	+ (Dai et al. 2008)	+	+ (Dai et al. 2008; Kuang et al. 2010; Ma et al. 2014)	+ (Dai et al. 2008; Kuang et al. 2010; Ma et al. 2014)	5 μM (Dai et al. 2008; Kuang et al. 2010; Ma et al. 2014)	+ (Dai et al. 2008; Kuang et al. 2010; Ma et al. 2014)	4.2 μM (Midgley et al. 2007)
	Neratinib	P-gp	NSCLC	+ (Zhao et al. 2012)	+ (Zhao et al. 2012; Hegedus et al. 2012)	ND	+ (Zhao et al. 2012)	+ (Zhao et al. 2012)	1 μM (Zhao et al. 2012)	+ (Zhao et al. 2012)	0.2 μM (Wong et al. 2009)

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RTK	TKI	ABC transporter(s) affected	Disease	Experimental data							
				IAAP labeling[a]	ATPase[b]	Transport substrate[c]	In vitro[d]	Reversal of MDR[e]	Reversal conc.[f]	In vivo[g]	Plasma conc.[h]
VEGFR	Apatinib	P-gp, ABCG2	Gastric carcinoma	+ (Mi et al. 2010)	+ (Mi et al. 2010)	ND	+ (Mi et al. 2010)	+ (Mi et al. 2010)	3 μM (Mi et al. 2010)	+ (Mi et al. 2010)	0.270 μM (Mi et al. 2010)
	Axitinib	P-gp, ABCG2	Advanced renal-cell carcinoma	ND	ND	+ (Poller et al. 2011)	+ (Poller et al. 2011)	+ (Wang et al. 2012a)	1 μM (Wang et al. 2012)	+ (Poller et al. 2011)	0.072 μM (Chen et al. 2013)
	Cediranib	P-gp, ABCG2, MRP1	Glioblastoma Multiforme	ND	ND	+ (Tao et al. 2009)	+ (Tao et al. 2009)	+ (Tao et al. 2009)	1.5 μM (Tao et al. 2009)	+ (Tao et al. 2009; Wang et al. 2012b)	1.13 μM (Wang et al. 2012)
	Motesanib	P-gp, ABCG2	Solid tumors	ND	+ (Wang et al. 2014d)	ND	+ Wang et al. (2014)	+ Wang et al. (2014)	3 μM Wang et al. (2014)	ND	8 μM (Hong et al. 2014)
	Pazopanib	P-gp, ABCG2	Renal-cell carcinoma, soft-tissue sarcoma	ND	ND	+ Minocha et al. (2012)	+ Minocha et al. (2012)	ND	ND	+ Minocha et al. (2012)	10–20 μM (Hurwitz et al. 2009)
	Sorafenib	P-gp, ABCG2	Hepatocellular Carcinoma	ND	ND	+ (Lagas et al. 2010; Poller et al. 2011)	+ (Wei et al. 1023)	+ (Wei et al. 1023)	4 μM (Wei et al. 1023)	+ (Lagas et al. 2010; Poller et al. 2011)	15–20 μM (Abou-Alfa et al. 2006)
	Sunitinib	P-gp, ABCG2	Hepatocellular carcinoma and renal-cell carcinoma	+ (Shukla et al. 2009)	+ (Shukla et al. 2009)	+ (Oberoi et al. 2013)	+ (Shukla et al. 2009)	+ (Shukla et al. 2009)	10 μM (Shukla et al. 2009)	+ (Hu et al. 2009; Tang et al. 2012)	0.251 μM (Goodman et al. 2007)
	Telatinib	ABCG2	Advanced metastatic solid tumors	ND	+ (Sodani et al. 2014)	ND	+ (Sodani et al. 2014)	+ (Sodani et al. 2014)	1 μM (Sodani et al. 2014)	+ (Sodani et al. 2014)	0.36 – 1.7 μM (Strumberg et al. 2008)
	Tivozanib	P-gp, ABCG2	No clinical indication	ND	ND	ND	+ (Yang et al. 2014)	+ (Yang et al. 2014)	5 μM (Yang et al. 2014)	ND	0.025 μM (Niwakawa et al. 2013)
	Vandetanib	P-gp, ABCG2, MRP1	Medullary thyroid cancer	ND	ND	+ (Minocha et al. 2012b)	+ (Mi and Lou 2007; Minocha et al. 2012)	+ (Mi and Lou 2007; Zheng et al. 2009)	3 μM (Mi and Lou 2007; Zheng et al. 2009)	+ (Mi and Lou 2007; Minocha et al. 2012)	1.64 μM (de Boer et al. 2009)

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Table 1 (continued)

RTK	TKI	ABC transporter(s) affected	Disease	Experimental data							
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FGFR	PD173074	P-gp	Research purposes only	+ (Patel et al. 2013)	+ (Patel et al. 2013)	ND	+ (Patel et al. 2013)	+ (Patel et al. 2013)	5 μM (Patel et al. 2013)	–	ND
Src/Abl kinase inhibitor	Saracatinib	P-gp	Ovarian cancer	+ (Liu et al. 2013)	+ (Liu et al. 2013)	ND	+ (Liu et al. 2013)	+ (Liu et al. 2013)	5 μM (Liu et al. 2013)	+ (Liu et al. 2013)	0.06–0.819 μM (Baselga et al. 2010)
p38 mitogen-activated protein kinase	BIRB796	P-gp, ABCG2, MRP1	Prevents mouse models of collagen-induced arthritis	ND	+ (He et al. 2013)	ND	+ (He et al. 2013)	+ (He et al. 2013)	10 μM (He et al. 2013)	+ (He et al. 2013)	0.74–7.38 μM (Branger et al. 2002)
Aurora kinase inhibitor	CCT129202.	P-gp, ABCG2	Research purposes only	ND	+ (Cheng et al. 2012)	ND	+ (Cheng et al. 2012)	+ (Cheng et al. 2012)	0.5 μM (Cheng et al. 2012)	+ (Cheng et al. 2012)	ND
Janus kinase 2 (JAK2)	CEP-33779	P-gp	Colorectal cancer	ND	+ (Tang et al. 2014)	ND	+ (Tang et al. 2014)	+ (Tang et al. 2014)	1.5 μM (Tang et al. 2014)	+ (Tang et al. 2014)	0.04–0.129 μM (Lu et al. 2011)
c-KIT, FAK, FGFR, PDGFR	Masitinib	ABCG2, MRP7	GIST	ND	ND	ND	+ (Kathawala et al. 2014a, b)	+ (Kathawala et al. 2014a, b)	2.5 μM (Kathawala et al. 2014a, b)	+ (Kathawala et al. 2014a, b)	0.921 μM (Bellamy et al. 2009)
Insulin-like growth factor 1	Linsitinib	P-gp, ABCG2, MRP7	Locally advanced and metastatic adrenocortical Carcinoma	ND	ND	ND	+ (Kathawala et al. 2014)	+ (Kathawala et al. 2014)	2 μM (Kathawala et al. 2014)	ND	2.6 μM (Mulvihill et al. 2009)
Anaplastic lymphoma kinase	Crizotinib	P-gp, ABCG2	NSCLC	ND	ND	+ (Chuan Tang et al. 2014)	+ (Zhou et al. 2012; Chuan Tang et al. 2014)	+ (Zhou et al. 2012)	1.5 μM (Zhou et al. 2012)	+ (Zhou et al. 2012)	0.6 μM (Chuan Tang et al. 2014; Li et al. 2011)

(continued)

Table 1 (continued)

RTK	TKI	ABC transporter(s) affected	Disease	Experimental data							
				IAAP labeling[a]	ATPase[b]	Transport substrate[c]	In vitro[d]	Reversal of MDR[e]	Reversal conc.[f]	In vivo[g]	Plasma conc.[h]
B-Raf	Vemurafenib	P-gp, ABCG2	Melanoma	+ (Wu et al. 2013)	+ (Durmus et al. 2012)	+ (Mittapalli et al. 2012; Wu and Ambudkar 2014)	+ (Mittapalli et al. 2012; Durmus et al. 2012)	+ (Durmus et al. 2012)	10 μM (Wu et al. 2013)	+ (Durmus et al. 2012)	9.6 μM (Grippo et al. 2014)
FLT3, PDGFR, and c-KIT	Tandutinib	ABCG2, MRP7	AML	+ (Zhao et al. 2013; Deng et al. 2013)	+ (Zhao et al. 2013; Deng et al. 2013)	+ (Yang et al. 2010)	+ (Zhao et al. 2013; Deng et al. 2013)	+ (Zhao et al. 2013; Deng et al. 2013)	3 μM (Zhao et al. 2013; Deng et al. 2013)	+ (Yang et al. 2010)	0.7 μM (DeAngelo et al. 2006)
Pim kinase inhibitor	SGI-1776	P-gp, ABCG2	Research purposes only	+ (Natarajan et al. 2013)	+ (Natarajan et al. 2013)	ND	+ (Natarajan et al. 2013)	+ (Natarajan et al. 2013)	1 μM (Natarajan et al. 2013)	ND	ND
FLT3	Crenolanib	P-gp	GIST	+ (Mathias et al. 2015)	+ (Mathias et al. 2015)	ND	+ (Mathias et al. 2015)	+ (Mathias et al. 2015)	0.5 μM (Mathias et al. 2015)	ND	0.7 μM (Galanis et al. 2014)
FLT3	Quizartinib	P-gp, ABCG2	AML	+ (Mathias et al. 2015)	+ (Mathias et al. 2015)	ND	+ (Mathias et al. 2015)	+ (Mathias et al. 2015)	10 μM (Mathias et al. 2015)	ND	26 μM (Cortes et al. 2013)

The table summarizes the TKIs reported to interact with ABC transporters, listing the key interactions, both biochemical and physiological, along with the intended primary physiological target RTK. (References are denoted in parentheses; *ND* Not-determined)

[a]TKIs that interact with P-gp and ABCG2 at the drug-binding pocket thereby inhibiting the binding of [^{125}I]-IAAP (Except erlotinib and PD173074) to P-gp and/or ABCG2

[b]TKIs that stimulate the ATPase activity of P-gp and ABCG2 in a concentration-dependent manner

[c]TKIs that have been demonstrated to be transport substrates for P-gp and ABCG2

[d]Reports indicating either inhibition of efflux function of ABC transporters and/or reversal of MDR in cells overexpressing ABC transporters in vitro

[e]TKIs that reverse MDR in cells in cytotoxicity assays

[f]Maximum concentration of TKIs reported to reverse MDR in cells overexpressing ABC transporters in vitro

[g]Studies conducted using a combination of TKI or alone in vivo to study tumor progression in tumor xenografts or study the body distribution of TKIs

[h]Plasma concentration of TKIs reported in mice during pre-clinical studies or in humans during clinical studies

drug–drug interactions (Fig. 1). This is especially important when drugs such as digoxin and cyclosporine A, which are substrates of ABC drug transporters and have a narrow therapeutic window, are concomitantly administered with TKIs. Therefore, the plasma concentration of such drugs should be closely monitored when administered with TKIs that inhibit the function of ABC drug transporters. Considering these interactions, the study of mechanistic details of how TKIs interact with transporters could be beneficial to develop a new generation of TKIs that show minimal interaction with these transporters.

In the following section, we discuss individual classes of RTKs and their respective inhibitors, with specific details on their biochemical interactions with ABC drug transporters, using substrate-binding and ATP hydrolysis assays (Fig. 2). We also elaborate on how functional activity of ABC transporters influences the pharmacokinetics of TKIs and toxicity profiles and discuss possible therapeutic implications of these interactions.

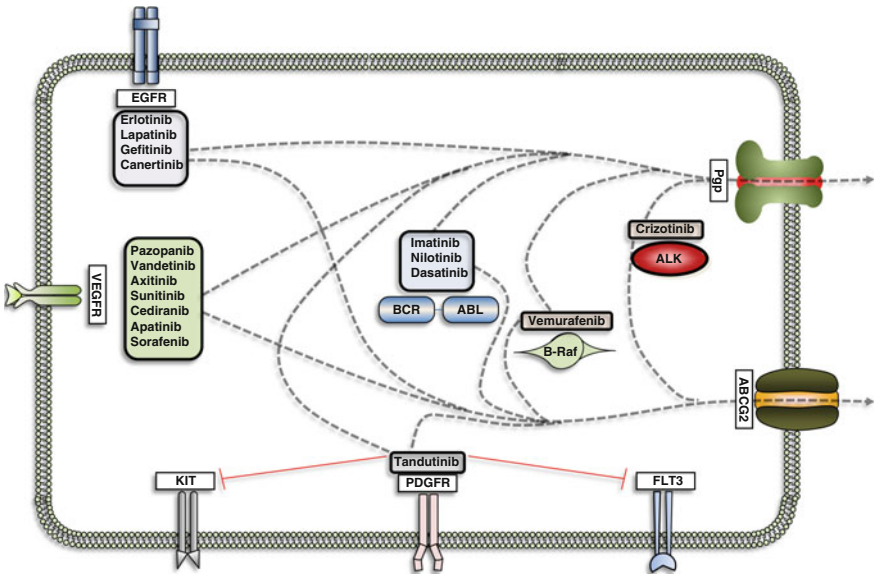

Fig. 1 Interplay between TKIs and ABC drug transporters. The inhibition of oncogenic RTKs by a single TKI or a combination of TKIs has resulted in improved survival rates among cancer patients. However, resistance to TKIs either by mutation in the kinase domains of target receptors or by upregulation of efflux transporters, such as P-gp or ABCG2, results in decreased therapeutic effects. This figure shows the selected tyrosine kinase targets for the TKIs that are transported by both P-gp and ABCG2 in vitro and in vivo, ultimately resulting in alteration in either brain or plasma pharmacokinetics and overall reduction in therapeutic effects

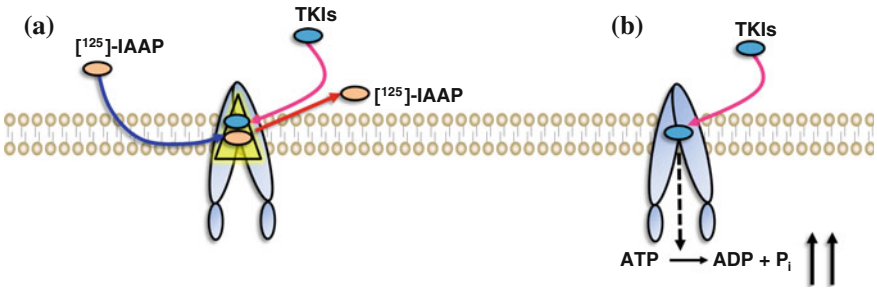

Fig. 2 Biochemical assays to determine direct interaction of TKIs with ABC transporters. **a** [^{125}I]-IAAP (IAAP) has been shown to directly bind to ABC drug transporters (P-gp and ABCG2) and covalently crosslink with residues lining the drug-binding pocket of these transporters upon exposure to UV light. IAAP is a substrate for both P-gp and ABCG2 and TKIs known to interact with both transporters displace IAAP in a concentration-dependent manner because they compete (*pink arrows*) for the same binding site (*yellow triangle*) on the ABC transporters (*light blue*) as IAAP, resulting in its displacement (*red arrow*). **b** TKIs, similar to other substrates, upon binding to the drug-binding pocket stimulate vanadate-sensitive ATPase activity of P-gp and ABCG2. These two biochemical assays are used to demonstrate direct interaction of TKIs with ABC drug transporters including P-gp, ABCG2, and MRP7 (IAAP labeling not demonstrated for this transporter)

Breakpoint Cluster-Abelson (BCR-ABL) Kinase Inhibitors

The BCR-ABL oncoprotein is characterized by its constitutive tyrosine kinase activity leading to malignant transformation of hematopoietic stem and progenitor cells. Imatinib became the first small molecule target-based therapeutic to receive approval by the FDA for the treatment of CML or acute lymphoblastic leukemia (ALL) in the late 1990s. Imatinib was the most potent inhibitor of BCR-ABL tyrosine kinase, targeting rapidly proliferating myeloid cells harboring the BCR-ABL fusion protein, with little effect on surrounding cells (Druker et al. 2001). Targets of imatinib also include platelet-derived growth factor receptor (PDGFR) and stem cell factor/c-kit. Resistance to imatinib treatment was soon observed in a subset of patients bearing mutations in the BCR-ABL fusion protein that blocks imatinib binding (Gorre et al. 2001). Structural exploration of TKIs targeting the BCR-ABL kinase led to several drugs that showed improved activity in patients with more than 50 mutations in BCR-ABL and fewer side effect profiles. However, cancer cells often evolve to resist the action of these TKIs, which then show poor activity (Gorre et al. 2001; Talpaz et al. 2006; Kantarjian et al. 2006). Of the several resistance mechanisms involved, overexpression of P-gp was one such mechanism responsible for the development of resistance to imatinib (Mahon et al. 2000, 2003; Peng et al. 2012). Furthermore, modulation of P-gp function either by its substrate cyclosporine A or by silencing of its expression in imatinib-resistant CML cell lines restored imatinib sensitivity (Illmer et al. 2004; Rumpold et al. 2005). Biochemical evidence confirmed high-affinity interaction of imatinib with P-gp using vanadate-sensitive ATPase assays and by evaluating its effect on the

binding of the P-gp photoaffinity substrate [^{125}I]-iodoarylazidoprazosin (IAAP) to the drug-binding pocket of this transporter (Fig. 2) (Shukla et al. 2008a). In addition, K562/VCR xenografts expressing both BCR-ABL and P-gp treated with imatinib in combination with daunorubicin or vincristine showed suppressed tumor growth (Gao et al. 2006). Reports describing the interaction of imatinib with ABCG2 soon followed. Houghton et al. first demonstrated that imatinib reversed ABCG2-mediated MDR, but was not a substrate of the transporter (Houghton et al. 2004). However, a systematic study by Breedveld et al. observed that imatinib could inhibit the transport of methotrexate by ABCG2 (Breedveld et al. 2004); in addition, the vectorial transport of imatinib in polarized epithelial cells was also demonstrated. Furthermore, we reported that imatinib interacts with ABCG2 and stimulates ATPase activity along with displacement of IAAP binding to the drug-binding site of ABCG2, thus confirming its direct interaction with ABCG2 (Shukla et al. 2008a). We also demonstrated that imatinib does not bind to the ATP sites or nucleotide-binding domains (NBDs) of P-gp or ABCG2, suggesting that the active ATP sites in kinases are different from those in ABC transporters (Shukla et al. 2008a). A recent study evaluated the use of COX-2 for targeting ABC transporters. Elevated expression of COX-2 was reported to be primarily responsible for the upregulation of ABC transporters such as MRP1, MRP2, MRP3 ABCA2, and ABCG2 in K562-imatinib resistant cells, thereby contributing toward the development of resistance. Combined treatment with imatinib and celocoxib proved to be beneficial, as K562 imatinib-resistant cells were rendered sensitive due to the downregulation of ABC transporters (Dharmapuri et al. 2015).

Nilotinib, dasatinib, ponatinib, and bosutinib—the second generation of BCR-ABL TKIs—have been shown to interact with P-gp and ABCG2, reversing MDR due to the action of these transporters in cancer cells (Brendel et al. 2007; Shukla et al. 2011; Tiwari et al. 2009; Hegedus et al. 2009; Sen et al. 2012). P-gp and ABCG2-expressing rat brain endothelial capillaries displayed decreased accumulation of BODIPY-conjugated nilotinib and [^{3}H]-nilotinib was also transported by P-gp in polarized LLC-PK1 cells. (Shukla et al. 2011). In addition to this, nilotinib significantly potentiates the anticancer activity of paclitaxel in ABCB1 and doxorubicin in ABCG2 tumor xenograft models (Tiwari et al. 2013). Hegedus et al. demonstrated a reduction in intracellular accumulation of nilotinib and dasatinib in both P-gp and ABCG2-overexpressing K562 cells (Hegedus et al. 2009), thereby suggesting that these TKIs are indeed transport substrates of the transporters. Biochemical evidence also affirms the high affinity of nilotinib, ponatinib, and dasatinib toward P-gp and ABCG2 (Sen et al. 2012; Dohse et al. 2010). K562 cells expressing P-gp and ABCG2 both exhibit resistance toward nilotinib, ponatinib, and dasatinib-mediated toxicity (Hegedus et al. 2009; Sen et al. 2012; Dohse et al. 2010). It was seen that all these TKIs exhibit higher affinity toward ABCG2 as compared to P-gp (Shukla et al. 2008a; Hegedus et al. 2009; Sen et al. 2012; Dohse et al. 2010). However, bosutinib was seen to weakly interact with P-gp and ABCG2 at micromolar concentrations (Hegedus et al. 2009). In addition to P-gp and ABCG2, imatinib, nilotinib, and ponatinib also interact with MRP7 to reverse resistance to paclitaxel in MRP7-overexpressing cells in vitro (Shen et al. 2009).

Epidermal Growth Factor Receptor (EGFR) Inhibitors

In 1962, Stanley Cohen isolated and characterized a protein from mouse salivary glands that induces eye-lid opening and tooth eruption in mice (Cohen 1962). The same protein was later shown to stimulate the proliferation of epithelial cells and hence was named the epidermal growth factor (EGF) (Cohen 1965). Subsequent work on the protein led to the identification of the EGF receptor (EGFR) (Carpenter et al. 1975), and its characterization as a RTK. Over a decade later, reports began to describe the overexpression of EGFR in several epithelial tumors, thus suggesting the role of dysregulated EGFR signaling (Ullrich et al. 1984; Libermann et al. 1984, 1985a, b).

Gefitinib was the first reversible EGFR TKI approved by the FDA in 2003 for use against non-small-cell lung cancer (NSCLC) (Wakeling et al. 1996). With the development of targeted TKIs, the identification of either acquired or intrinsic resistance soon followed, with ABC transporters being the prime candidates (Jackman et al. 2010). Gefitinib was reported to interact with P-gp and ABCG2; however, it possesses higher affinity for ABCG2 as measured by its ability to stimulate the ATPase activity of this transporter (Ozvegy-Laczka et al. 2004). ABCG2-transduced cells conferred increased resistance to gefitinib-mediated cytotoxicity (Yanase et al. 2004; Lemos et al. 2009). Treatment with gefitinib enhanced the oral bioavailability of irinotecan in mice after simultaneous oral administration. Multidrug-resistant lung cancer cells expressing P-gp reportedly exhibited reduced resistance to substrate chemotherapeutic drugs when combined with gefitinib (Kitazaki et al. 2005). Patients harboring the Q141K mutation in ABCG2 have a transporter with reduced expression at the cell surface of renal tubules, and administration of gefitinib resulted in diarrhea with no changes in gefitinib pharmacokinetics, suggesting involvement of ABCG2 in gefitinib disposition (Cusatis et al. 2006).

Erlotinib, another reversible EGFR-TKI, was soon reported to inhibit P-gp and ABCG2-mediated MDR in KB-C2 and HEK293 cells expressing P-gp and ABCG2, respectively, along with decreasing the efflux of $E_2$17βG and methotrexate in ABCG2-expressing membrane vesicles (Shi et al. 2007). Erlotinib also stimulated the ATPase activity of P-gp and ABCG2; however, it did not inhibit the labeling of either transporter with IAAP. Erlotinib was shown to be transported by both P-gp and ABCG2, and the absence of either transporter significantly altered the pharmacokinetics of orally administered erlotinib (Marchetti et al. 2008). In addition to P-gp and ABCG2, erlotinib also reversed MRP7-mediated MDR in vitro (Kuang et al. 2010).

Lapatinib, an EGFR inhibitor approved for the treatment of breast cancer in 2007, was reported to be a substrate of both P-gp and ABCG2 (Burris et al. 2005). Lapatinib increased the cytotoxicity of paclitaxel and doxorubicin in P-gp- and ABCG2-overexpressing cancer cells, respectively, and inhibited the tumor growth of xenografts overexpressing P-gp when combined with paclitaxel (Dai et al. 2008). Lapatinib exhibits a higher affinity for ABCG2 compared to P-gp (based on ATPase

activity) and it displaced the binding of IAAP to both transporters with similar IC_{50} values (Dai et al. 2008). Additionally, lapatinib was shown to reverse MRP1 and MRP7-mediated MDR (Kuang et al. 2010; Ma et al. 2014). Interestingly, studies performed on elacridar-treated rats showed no significant change in the systemic exposure of orally administered lapatinib (Polli et al. 2008) and its exposure to the brain remained the same among wild-type mice dosed with intravenous lapatinib compared to *mdr1a/b*$^{-/-}$ mice dosed similarly (Polli et al. 2008).

Neratinib, a potent, orally administered inhibitor of the EGFR family of receptors, has the ability to irreversibly inhibit multiple EGFR receptors and effectively inhibit the proliferation of EGFR- and HER2-expressing cells that are resistant to treatment with first-generation EGFR inhibitors (Rabindran et al. 2004; Tsou et al. 2005; Kwak et al. 2005). Neratinib is currently undergoing clinical investigation for its anticancer effect against metastatic breast cancer and NSCLC (Kwak et al. 2005; Wong et al. 2009; Shimamura et al. 2006; Bose et al. 2013; Ji et al. 2006). Zhao et al. reported that neratinib reversed P-gp-mediated MDR in both drug-selected and transfected cell lines overexpressing P-gp (Zhao et al. 2012). It also increased the intracellular accumulation of the P-gp fluorescent substrates rhodamine123 and doxorubicin in P-gp-expressing cells derived from patients and in drug-selected P-gp-overexpressing cancer cells. Neratinib stimulated the ATPase activity of P-gp and inhibited photolabeling of this transporter with IAAP in a concentration-dependent manner (Zhao et al. 2012). Tumor xenografts overexpressing P-gp were significantly reduced in size when treated with neratinib compared to the vehicle-treated control xenografts. Contrary to its effect on P-gp activity, neratinib did not have any effect on ABCG2 functional activity (Hegedus et al. 2012).

Afatinib, an irreversible EGFR and HER2 inhibitor, potently suppresses the kinase activity of wild-type as well as activated mutant cells (Bean et al. 2008). It is currently approved as a first-line treatment for patients with metastatic NSCLC with wild-type or mutant EGFR. Afatinib treatment in vitro was found to enhance the chemotherapeutic efficacy in side-population cells by decreasing ABCG2 expression with increasing levels of DNA methyltransferase and methylating the ABCG2 promoter, leading to downregulation of ABCG2 (Wang et al. 2014a). Along with reduction in the surface expression of ABCG2, afatinib also reversed MDR in S1-M1-80 mitoxantrone-resistant colorectal cancer cells overexpressing ABCG2 both in vitro and in vivo (Wang et al. 2014b).

Icotinib, an orally active, potent EGFR TKI has been approved by the Chinese Food and Drug Administration as a second- or third-line treatment for patients with advanced NSCLC (Hu et al. 2012; Shi et al. 2013; Tan et al. 2013). Icotinib was recently shown to interact with ABCG2 and reverse ABCG2-mediated MDR in H460/MX20 NSCLC ABCG2-overexpressing drug-resistant cells and HEK293 cells transfected with ABCG2. Additionally, icotinib increased ATPase activity and inhibited the IAAP labeling of ABCG2, suggesting its direct interaction with the transporter. ABCG2-overexpressing tumor xenografts showed reduced growth rates when treated with a combination of icotinib and topotecan, which is a substrate of ABCG2 (Wang et al. 2014b).

Canertinib, a small molecule irreversible inhibitor of pan-EGFR, was observed to increase the intracellular accumulation and subsequent toxicity of topotecan and SN-38 (active metabolite of irinotecan) in ABCG2-overexpressing cells (Erlichman et al. 2001). Canertinib was reported to be actively effluxed by ABCG2, thereby suggesting its role in the development of resistance to this TKI in cancer cells (Erlichman et al. 2001). Consistent with these findings, Minocha et al. (2012a) observed an increase in the brain penetration of pazopanib in mice treated with either erlotinib or canertinib.

AST1306, a small molecule irreversible inhibitor targeting the EGFR and HER2 receptors, is currently under clinical investigation for the treatment of advanced solid tumors. It was found to have a role in reversing ABCG2-mediated MDR in H460/MX20 and HEK293 cells overexpressing ABCG2 with minimal interaction with P-gp (Zhang et al. 2014a). Increased accumulation of [^{3}H]-mitoxantrone and stimulation of ATPase activity of ABCG2 in the presence of AST1306 suggest its ability to modulate the function of this transporter (Zhang et al. 2014a).

AG1478, another TKI, was developed as a highly potent EGFR inhibitor but is primarily used now as a research tool (Gazit et al. 1996). This inhibitor reversed MDR in KB-C2 cells overexpressing P-gp and HEK293 cells transfected with ABCG2 in vitro (Shi et al. 2009). AG1478 at non-toxic concentrations was able to increase the intracellular accumulation of [^{3}H]-paclitaxel and [^{3}H]-mitoxantrone in cells overexpressing P-gp and ABCG2, respectively. In addition, AG1478 increased the accumulation of $E_2$17βG and methotrexate in inside-out membrane vesicles (Shi et al. 2009). The ATPase activity of both P-gp and ABCG2 was stimulated with increasing concentrations of AG1478, with more affinity toward ABCG2 than P-gp. AG1478 also reduced the labeling of P-gp and ABCG2 with IAAP due to its interaction at the drug-binding site of these transporters (Shi et al. 2009).

ARRY-334543, a potent selective reversible inhibitor of epidermal growth receptor (ErbB1 and ErbB2), is currently under clinical investigation for the treatment of metastatic breast cancer (Lee et al. 2009). ARRY-334543 reversed ABCG2-mediated MDR in cancer cells, stimulated the ATPase activity, and inhibited the binding of IAAP to ABCG2 (Wang et al. 2014c). ARRY-334543 also partially reversed P-gp- and MRP7-mediated MDR (Wang et al. 2014c).

Vascular Endothelial Growth Factor Receptor (VEGFR) Inhibitors

Angiogenesis, the process of forming new blood vessels from pre-existing blood vessels and vascular cells, plays an important role in several physiological processes such as embryogenesis and blood-vessel formation and is associated with cardiovascular diseases, ulcers, rheumatoid arthritis, atherosclerosis, macular degeneration, and most importantly cancer (Dvorak 2005). Induction of angiogenesis, or as

widely known, the angiogenic switch is characterized by the upregulation of pro-angiogenic factors including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) (Hanahan and Folkman 1996). The VEGF receptors (VEGFR) are a family of three receptor tyrosine kinases (VEGFR 1, 2, and 3) that recognize fiveVEGF ligands (Olsson et al. 2006). Due to structural similarities, drugs targeting VEGFR also show activity toward PDGFR and c-KIT receptors (Hubbard and Till 2000).

Sunitinib was among the first small molecule TKIs to be approved for the treatment of unresectable hepatocellular carcinoma and advanced renal-cell carcinoma, for first-line and second-line treatment of metastatic renal-cell carcinoma and for the treatment of imatinib-resistant GIST. Sunitinib inhibits VEGFR, PDGFR, and c-Kit kinases. We demonstrated that sunitinib interacts with both P-gp and ABCG2, as it inhibited the efflux of fluorescent substrates in cells overexpressing these transporters (Shukla et al. 2009). In vitro assays revealed that sunitinib partially reversed MDR mediated by P-gp. However, it completely reversed ABCG2-mediated MDR in KB-V1 and HEK293 cells expressing P-gp and ABCG2 by interacting at the substrate-binding pockets of the transporters (Shukla et al. 2009). There was a two-fold increase in brain accumulation of sunitinib (dose-20 mg/kg) in *mdr1a/b*$^{-/-}$ mice compared to wild-type P-gp-expressing mice (Hu et al. 2009). However, Tang et al. demonstrated that upon co-administration of oral sunitinib (10 mg/kg) with elacridar, another inhibitor of both P-gp and ABCG2, there was a marked increase in the brain accumulation of sunitinib (Tang et al. 2012). These observations were similar to what was observed in *mdr1a/b*$^{-/-}$, *abcg2*$^{-/-}$ mice. Chee et al. reported an increase in the tissue distribution of sunitinib upon co-administration with ketoconazole, an antifungal agent having potent inhibitory action toward CYP3A4 and P-gp (Chee et al. 2015). It was also reported that the Q141K ABCG2 polymorphism, often encountered in Asian populations, results in decreased surface expression of this transporter (Sparreboom et al. 2005; Thomas et al. 2009). A two-fold increase in the steady-state plasma concentration of orally administered sunitinib was observed in patients harboring the Q141K mutation in ABCG2, thereby recommending a dose adjustment for such patients (Mizuno et al. 2014). Thus, adjustment of the dose of sunitinib in individuals expressing polymorphic variants of ABC drug transporters is necessary to avoid its harmful toxic effects.

Sorafenib, a multikinase inhibitor of VEGFR, PDGFR, and B-type Raf Kinase (B-Raf), has become the standard chemotherapeutic agent for the treatment of hepatocellular carcinoma worldwide (Bruix et al. 2011; Verslype et al. 2012; Ren et al. 2015). Sorafenib has been reported to reverse P-gp-mediated MDR in cancer cells along with reduced expression of P-gp (Wei et al. 1023). A twofold increase in the brain penetration of sorafenib in *mdr1a/b*$^{-/-}$ mice was observed (Huang and Tang 2003). Lagas et al. (2010) demonstrated moderate sorafenib transport by P-gp-expressing polarized cells. However, it was transported more efficiently by ABCG2-expressing polarized cells. Although there was no change in systemic exposure of sorafenib in *mdr1a/b*$^{-/-}$, *abcg2*$^{-/-}$ mice, there was a 4.3-fold increase

in the brain accumulation of sorafenib in *abcg2*$^{-/-}$ mice and a 9.3-fold increase in *mdr1a/b*$^{-/-}$, *abcg2*$^{-/-}$ mice as compared to the wild-type mice (Lagas et al. 2010). Sorafenib was a major determinant of ABCG2-mediated transport at lower concentrations. However, higher concentrations of sorafenib showed saturation of this effect in MDCKII polarized cells transfected with both P-gp and ABCG2 (Poller et al. 2011). A recent report indicated the results of Phase I/II clinical trials involving combination therapy for patients with advanced K-ras-mutated colorectal cancer treated with a combination of sorafenib and irinotecan to have improved outcomes mainly due to inhibition of ABCG2 efflux function by sorafenib (Gongora 2014).

Pazopanib, an oral angiogenesis inhibitor that blocks the signaling of VEGFR, PDGFR, and c-KIT, is approved for the treatment of renal-cell carcinoma and soft-tissue sarcoma (Sternberg et al. 2010; van der Graaf et al. 2012). Minocha et al. reported pazopanib to be a high-affinity substrate for ABCG2 with moderate interaction with P-gp. Inhibition of P-gp with zosuquidar or ABCG2 with Ko143 did not alter brain accumulation of pazopanib. However, treatment with elacridar significantly increased brain penetration of pazopanib (Minocha et al. 2012a).

Axitinib, approved by the FDA in 2012 for the treatment of advanced renal-cell carcinoma, is a potent VEGFR, PDFGR, and c-KIT inhibitor. Axitinib is reported to be a good substrate of P-gp as compared to ABCG2 in studies using polarized cells expressing P-gp or ABCG2 (Poller et al. 2011). The plasma concentration of axitinib remained unchanged in *mdr1a/b*$^{-/-}$, *abcg2*$^{-/-}$ mice. However, there was a significant increase in the brain penetration of axitinib in *mdr1a/b*$^{-/-}$, *abcg2*$^{-/-}$ mice, with major differences attributed to the absence of P-gp as compared to ABCG2 (Poller et al. 2011). In addition to reversing ABCG2-mediated MDR in A549 NSCLC side-population cells, it also enhanced the cytotoxicity of topotecan or irinotecan in side-population cells isolated from A549 cells (Wang et al. 2012a). Another study reported axitinib to be a weaker substrate of ABCG2 than P-gp (Reyner et al. 2013).

Cediranib, an orally active inhibitor identified to target VEGFR, PDGFR, and c-KIT, is currently undergoing clinical trials for treatment of glioblastoma multiforme. Tao et al. reported the reversal of P-gp and MRP1-mediated drug transport, using a combination of P-gp and MRP1 substrates with cediranib (Tao et al. 2009). Wang et al. demonstrated that cediranib is transported by both P-gp and ABCG2 in vivo with higher affinity of cediranib toward P-gp and with moderate changes in cediranib plasma concentration in knock-out mice lacking both transporters (Wang et al. 2012b). However, there was higher brain accumulation of cediranib in *mdr1a/b*$^{-/-}$, *abcg2*$^{-/-}$ mice, thus suggesting limited brain accumulation of cediranib primarily due to P-gp efflux function.

The FDA approved the use of vandetanib, a VEFGR, PDGFR, and c-KIT kinase inhibitor, for the treatment of symptomatic or progressive medullary thyroid cancer in patients with unresectable, locally advanced, or metastatic disease. Vandetanib was reported to inhibit P-gp-mediated MDR in MCF7/Adr (doxorubicin-selected) breast cancer cells by blocking its efflux function at physiologically achievable concentrations (Mi and Lou 2007). Consequently, vandetanib increased the

intracellular accumulation of rhodamine 123 and doxorubicin in P-gp-overexpressing cells; it also stimulated the ATPase activity of P-gp (Mi and Lou 2007). Furthermore, vandetanib was shown to reverse MRP1- and ABCG2-mediated MDR, by blocking their efflux function (Zheng et al. 2009). Brain accumulation of vandetanib was limited by the presence of P-gp and ABCG2 and co-administration of vandetanib with elacridar resulted in a five-fold increase in vandetanib brain accumulation (Minocha et al. 2012b).

Tivozanib, an orally active potent VEGFR inhibitor, was reported to reverse ABCG2 and P-gp-mediated MDR in HEK293 cells overexpressing ABCG2 and KB-C2 cells overexpressing P-gp (Yang et al. 2014). It stimulated the ATPase activity of both transporters and inhibited the binding of IAAP to both P-gp and ABCG2 (Yang et al. 2014). The effect of tivozanib on other ABC transporters remains to be determined.

Nintedanib, an inhibitor of VEGFR, FGFR, PDGFR, and c-KIT, has recently been approved for the treatment of idiopathic pulmonary fibrosis. Xiang et al. demonstrated the effect of nintedanib on reversal of the P-gp-mediated MDR in cancer cells, whereby it resensitized the cytotoxic effects of P-gp substrates in P-gp-overexpressing cancer cells (Xiang et al. 2011). Nintedanib inhibited the ATPase activity of P-gp thereby suggesting its interaction with P-gp.

Telatinib, another TKI inhibiting VEGFR, PDGFR, and c-KIT, is still under clinical investigation (Eskens et al. 2009). Telatinib reversed ABCG2-mediated MDR in H460/MX20 and HEK293 cells overexpressing ABCG2 by inhibiting its efflux function both in vitro and in vivo (Sodani et al. 2014). Telatinib stimulated the ATPase activity of ABCG2 and inhibited the ABCG2-mediated transport of $E_2$17βG in ABCG2 (Sodani et al. 2014). Administering telatinib in combination with doxorubicin led to a significant decrease in the size of tumor xenografts expressing ABCG2 as compared to vehicle control or the group treated with doxorubicin alone (Sodani et al. 2014).

Motesanib, an orally bioavailable VEGFR, PDGFR, and c-KIT inhibitor, is currently being evaluated for its antitumor activity (Polverino et al. 2006; Coxon et al. 2009, 2012). Motesanib inhibited the efflux function of P-gp and resensitized P-gp-expressing cells to anticancer drugs that are substrates of this transporter (Wang et al. 2014d). Motesanib stimulated the ATPase activity of P-gp (Wang et al. 2014d) and partially reversed ABCG2-mediated MDR without having any effect on MRP1-overexpressing cells (Wang et al. 2014d).

Apatinib, an orally bioavailable VEGFR, PDGFR, and c-KIT inhibitor, is currently undergoing clinical trials for the treatment of advanced gastric carcinoma (Geng and Li 2015; Mi et al. 2010; Li et al. 2013). Apatinib increased the intracellular accumulation of rhodamine 123 and doxorubicin in multidrug resistant cells overexpressing P-gp and ABCG2 in addition to stimulating the ATPase activity of these transporters (Mi et al. 2010). Moreover, apatinib inhibited the binding of IAAP to both P-gp and ABCG2 (Mi et al. 2010). Tumor xenografts overexpressing P-gp showed reduced growth when treated with a combination of paclitaxel and apatinib (Mi et al. 2010).

Miscellaneous TKIs

PD173074, a potent fibroblast growth factor receptor inhibitor (FGFR), exhibits a high degree of complementarity toward the kinase domain of FGFR1 (Mohammadi et al. 1998). Earlier, we reported PD173074 to selectively reverse P-gp-mediated MDR in KB-C2 and HEK293 cells overexpressing P-gp in vitro. PD173074 increased the intracellular accumulation of [^{3}H]-paclitaxel in intact cells and stimulated the ATPase activity of P-gp. Surprisingly PD173074 did not inhibit the photolabeling of P-gp with IAAP, thereby suggesting that even though PD173074 interacts with P-gp, as seen by its stimulatory effect on ATPase activity, it binds to a different site on the transporter (Patel et al. 2013). Recently, Anreddy et al. demonstrated that PD173074 reverses MRP7-mediated MDR in vitro in MRP7-expressing cells (Anreddy et al. 2014b).

Saracatinib, a highly selective, dual Src/Abl kinase inhibitor, is currently in a Phase II clinical trial for the treatment of ovarian cancer (Green et al. 2009). Saracatinib reversed P-gp-mediated MDR in HeLa/v200 cells, with no such effects observed in MRP1-overexpressing cancer cells (Liu et al. 2013). Saracatinib-treated MCF-7/Adr, HEK293/ABCB1, and HeLa/v200 cells showed increased intracellular accumulation of rhodamine 123 and doxorubicin and stimulated ATPase activity in crude membranes expressing P-gp. Furthermore, saracatinib decreased the binding of IAAP to P-gp, suggesting an interaction of saracatinib at the substrate-binding pocket of P-gp (Liu et al. 2013). In addition, saracatinib significantly attenuated the tumor growth of HeLa/v200 xenografts in vivo when administered orally along with paclitaxel (Liu et al. 2013).

BIRB796 is one of the most potent compounds that bind to p38-MAPK with high affinity. However, it indirectly competes with the binding of ATP and has been reported to suppress endotoxin-mediated TNFα production and prevent collagen-induced arthritis in mouse models (Ryoo et al. 2013). BIRB796 reversed P-gp-mediated MDR in MCF7/Adr and KB/v200 cells overexpressing P-gp with minimal reversal activity in MRP1 and ABCG2-overexpressing cancer cells. BIRB796 showed a biphasic effect on the ATPase activity of P-gp, stimulating the ATPase activity at lower concentrations and inhibiting it at higher concentrations (Ryoo et al. 2013). BIRB796 also increased the intracellular accumulation of rhodamine 123 and doxorubicin in P-gp-overexpressing cells (Ryoo et al. 2013).

CCT129202, a novel selective aurora kinase inhibitor with minimal activity against other kinases (Chan et al. 2007), was tested for its activity in reversing MDR in KB/v200, MCF7/Adr, and HL60/Adr cells (Cheng et al. 2012). CCT129202 significantly enhanced the cytotoxicity of substrate drugs and increased the intracellular accumulation of doxorubicin and rhodamine 123 in P-gp- and ABCG2-overexpressing cells. Interestingly, CCT129202 also potentiated the sensitivity of cancer stem-like cells to doxorubicin. In vivo studies with CCT129202 showed an increase in the inhibitory effects of vincristine and paclitaxel on P-gp-overexpressing KBv200 xenografts in nude mice and human esophageal cancer tissue overexpressing ABCB1 ex vivo, respectively (Cheng et al. 2012).

The ATPase activity of P-gp was inhibited with increasing concentrations of CCT129202 (Cheng et al. 2012).

CEP-33779 is a novel, highly selective and orally bioavailable, ATP-competitive, and small molecule Janus kinase 2 (JAK2) inhibitor with a favorable preclinical profile (Seavey et al. 2012). Tang et al. reported an increase in the cytotoxicity of P-gp substrate chemotherapeutics in P-gp-overexpressing cells when treated in combination with CEP-33779 (Tang et al. 2014). This was attributed to an increase in the intracellular accumulation of P-gp substrate drugs. Similarly, there was an increase in the ATPase activity of P-gp with increasing concentrations of CEP-33779. Tumor xenografts overexpressing P-gp showed a marked reduction in growth rate compared to vehicle-treated controls when treated with a combination of vincristine and CEP-33779 (Tang et al. 2014).

Masitinib mesylate (AB1010) is a novel, potent, and selective TKI targeting mainly wild-type and mutant c-Kit receptor, PDGFR, lymphocyte-specific kinase Lck, FGFR, and focal adhesion kinase (FAK) (Humbert et al. 2010). Masitinib is currently being evaluated in clinical studies for the treatment of gastrointestinal stromal tumors (Le Cesne et al. 2010; Campanella et al. 2013; Kim and Zalupski 2011). Masitinib has been reported to reverse MRP7- and ABCG2-mediated MDR in H460/MX20 and HEK293 cells overexpressing ABCG2 (Kathawala et al. 2014a, b). Masitinib increased the cytotoxicity of paclitaxel in MRP7-overexpressing HEK293 cells mainly due to an increase in paclitaxel intracellular accumulation (Kathawala et al. 2014b). In vivo tumor xenografts expressing MRP7 showed marked reduction in growth rate when treated with a combination of masitinib and paclitaxel, as compared to vehicle-treated control cells (Kathawala et al. 2014b). Another independent report demonstrated that masitinib enhanced the cytotoxic effects of ABCG2 substrates in vitro mainly due to an increase in the intracellular accumulation of ABCG2 substrates in the presence of masitinib (Kathawala et al. 2014a). Masitinib was also shown to reverse P-gp-mediated MDR to doxorubicin in canine lymphoid tumor cells (Zandvliet et al. 2013).

Linsitinib, an inhibitor of insulin-like growth factor 1 insulin receptor, is currently under clinical trials for locally advanced or metastatic adrenocortical carcinoma (Mulvihill et al. 2009). Linsitinib effectively reversed MDR-overexpressing ABCG2 and MRP7-transfected HEK293 cells (Zhang et al. 2014b). This effect was mainly due to an increase in the intracellular accumulation of [^{3}H]-mitoxantrone and [^{3}H]-paclitaxel in ABCG2- and MRP7-expressing cells upon treatment with linsitinib (Zhang et al. 2014b). Linsitinib moderately enhanced the cytotoxicity of P-gp substrate drugs with no such effect on MRP1-expressing cells (Zhang et al. 2014b).

Crizotinib, an orally bioavailable TKI, has been approved for the treatment of NSCLC in patients with the echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase gene (EML-ALK) translocations (Kwak et al. 2010; Christensen et al. 2007; McDermott et al. 2008). Crizotinib enhanced the cytotoxicity of P-gp substrate drugs in P-gp-expressing cells; this effect was mainly attributed to the inhibition of efflux of substrate drugs as seen by the reduction in rhodamine 123 and doxorubicin efflux in P-gp-overexpressing cells. In addition,

P-gp-overexpressing tumor xenografts showed marked reduction in growth rate when treated with a combination of crizotinib and paclitaxel (Zhou et al. 2012). Crizotinib has also been reported to be a good substrate for P-gp as compared to ABCG2 (Chuan Tang et al. 2014). Low dose crizotinib-treated *mdr1a/b*$^{-/-}$, *abcg2*$^{-/-}$ mice showed a twofold increase in plasma crizotinib concentration, whereas brain accumulation increased to 40-fold higher crizotinib levels (Chuan Tang et al. 2014). Surprisingly, high dose crizotinib resulted in a similar plasma profile of crizotinib in *mdr1a/b*$^{-/-}$, *abcg2*$^{-/-}$ mice as compared to wild type; however, there was a 70-fold increase in brain accumulation of crizotinib in these mice (Chuan Tang et al. 2014). This suggests that crizotinib accumulation in the brain could be enhanced in NCSLC patients upon combining treatment with P-gp and ABCG2-specific inhibitors.

Vemurafenib, targeting a specific mutation in the B-Raf protein tyrosine kinase, received approval in 2011 for the treatment of B-Raf mutant (V600E) melanomas (Flaherty et al. 2010; Johannessen et al. 2010; Nazarian et al. 2010). Mittapalli et al. (2012) demonstrated vemurafenib to be a substrate of P-gp and ABCG2 using polarized MDCKII cells expressing these transporters. Vemurafenib accumulation in the brain was limited due to the expression of P-gp and ABCG2 at the blood–brain barrier; this could be enhanced by co-administration of elacridar (Mittapalli et al. 2012). In another independent study, Durmus et al. showed that there was a sixfold increase in the plasma concentration and a 21.4-fold increase in the brain concentration of vemurafenib in *mdr1a/b*$^{-/-}$, *abcg2*$^{-/-}$ mice (Durmus et al. 2012). They also demonstrated increased vemurafenib brain penetration in mice treated with elacridar and vemurafenib co-treatment. Vemurafenib was found to stimulate the ATPase activity of P-gp and ABCG2 along with an inhibition of efflux of P-gp and ABCG2 in KB-V1 and MCF7-FLV-1000 cells overexpressing P-gp and ABCG2, respectively (Wu et al. 2013). These studies implicate the role of P-gp and ABCG2 in poor penetration of vemurafenib and resulting decreased efficacy of vemurafenib against metastatic brain melanomas, which can be enhanced via co-administration with inhibitors of ABC transporters. The contribution of ABC transporters toward vemurafenib resistance was recently reviewed in Wu and Ambudkar (2014).

Tandutinib, a potent FMS-like tyrosine kinase 3 (FLT3), PDGFR, and c-KIT inhibitor, also reverses ABCG2-mediated MDR in S1-M1-80 and HEK293 cells overexpressing ABCG2 by increasing the intracellular accumulation of anticancer agents, thereby enhancing their cytotoxic abilities in cells expressing ABCG2. It stimulated the ATPase activity of ABCG2 and inhibited the binding of IAAP to ABCG2 (Zhao et al. 2013). Yang et al. reported tandutinib to be a substrate of P-gp and ABCG2, resulting in decreased brain accumulation of tandutinib (Yang et al. 2010). A sevenfold increase in the mean plasma concentration of tandutinib was observed in rats treated with elacridar. Similarly, *mdr1a/b*$^{-/-}$, *abcg2*$^{-/-}$ mice showed a twofold increase in the plasma concentration of tandutinib and a 13-fold increase in brain accumulation was observed in these mice (Yang et al. 2010). Tandutinib is also reported to reverse MDR in MRP7-expressing cells (Deng et al. 2013).

Crenolanib, an FLT3 kinase inhibitor that also inhibits PDGFR, is currently undergoing clinical trials for the treatment of GIST in imatinib-resistant patients (Heinrich et al. 2012). Crenolanib was identified to be a substrate of P-gp, as observed by resistance to cytotoxicity in P-gp-overexpressing cells, which could be abolished by combining crenolanib with the P-gp transport inhibitor PSC-833 (valsopodar) (Mathias et al. 2015). This phenomenon was not observed in ABCG2- or MRP1-expressing cells. Crenolanib also stimulated the ATPase activity along with inhibition of the binding of IAAP to P-gp (Mathias et al. 2015).

Quizartinib, an FLT3 inhibitor, is currently under trial for the treatment of refractory AML (Ostronoff and Estey 2013). Quizartinib completely and partially inhibited ABCG2- and P-gp-mediated MDR in cancer cells, respectively, and increased the intracellular accumulation of substrates of ABCG2 and P-gp (Bhullar et al. 2013). Quizartinib stimulated the ATPase activity of both P-gp and ABCG2 at lower concentrations and inhibited this activity at higher concentrations (Bhullar et al. 2013). Quizartinib resensitized ABCG2-expressing cells to ABCG2 chemotherapeutic drugs in cancer cells. An increase in the uptake of ciprofloxacin in the presence of quizartinib was observed, suggesting an alteration in the pharmacokinetic parameters of drugs given in combination with quizartinib (Bhullar et al. 2013).

Another kinase inhibitor, SGI-1776, which mainly inhibits the activity of Pim kinase, was reported to interact with P-gp and ABCG2, as it increased the accumulation of P-gp and ABCG2 substrate chemotherapeutics, thereby resensitizing resistant cells (Natarajan et al. 2013). SGI-1776 also stimulated the ATPase activity of P-gp and ABCG2, and inhibited the binding of IAAP to P-gp and ABCG2. Surprisingly, SGI-1776 decreased the expression of P-gp and ABCG2 by inhibiting Pim1 kinase-mediated phosphorylation of these transporters, and possibly increasing their degradation by lysosomal and proteolytic degradation (Natarajan et al. 2013; Xie et al. 2010).

Collectively, the extensive studies detailed above and summarized in Table 1 suggest that while TKIs interact specifically with RTKs they also have off-target effects. They interact with ABC drug transporters, leading to altered pharmacokinetics in certain physiological or disease conditions, which might also result in potential drug–drug interactions. These non-targeted effects of TKIs can be eliminated if TKIs have minimal interactions with ABC drug transporters. Current mechanistic knowledge about how TKIs are specifically recognized by ABC drug transporters is limited. The structural information that could explain the mechanistic differences in the binding of TKIs to their respective kinases and transporters has not been addressed in detail. As summarized in Table 2, while several crystal structures of TKIs bound to active sites of their targets are available, similar information about structural aspects of TKIs with ABC transporters is not yet available. The following section discusses the biochemical and structural information on the interaction of TKIs with both kinases and ABC transporters.

Table 2 Known structures of TKIs in association with their targets

TKI[a]	Target kinases (Structure known; PDB code)	References
Afatinib	EGFR (4G5P, 4G5J)	Solca et al. (2012)
Axitinib	VEGFR-2 (4AG8)	McTigue et al. (2012)
Bosutinib	BCR-ABL (3UE4)	Levinson and Boxer (2012)
Crizotinib	ALK (2YFX, 4ANQ, 2XP2, 4ANS)	Huang et al. (2014), Cui et al. (2011)
Dasatinib	BCR-ABL (2GQG), PDGFR (2GQG)	Tokarski et al. (2006)
Erlotinib	EGFR (1M17)	Stamos et al. (2002)
Gefitinib	EGFR (4I22, 3UG2, 4I1Z)	Yoshikawa et al. (2013), Gajiwala et al. (2013)
Imatinib	BCR-ABL (3K5V, 2HYY, 3MS9, 3MSS, 1IEP, 3GVU), c-Src (2OIQ, 3OEZ)	Nagar et al. (2002), Cowan-Jacob et al. (2007), Seeliger et al. (2007), Zhang et al. (2010), Jahnke et al. (2010)
Lapatinib	EGFR (1XKK), ErBB4 (3BBT)	Qiu et al. (2008), Wood et al. (2004)
Motesanib	VEGFR (3EFL)	Protein Data Bank (PDB)
Neratinib	EGFR (3W2Q, 2JIV)	Yun et al. (2008)
Nilotinib	BCR-ABL (3CS9)	Weisberg et al. (2005)
Pazopanib	VEGFR-2 (3CJG, 3CJF)	Harris et al. (2008)
Ponatinib	BCR-ABL (3OXZ, 3OY3)	Zhou et al. (2011)
Sorafenib	VEGFR-2 (4ASD)	McTigue et al. (2012)
Sunitinib	VEGFR-2 (4AGD)	McTigue et al. (2012)
Tivozanib	VEGFR-2 (4ASE)	McTigue et al. (2012)

[a]List includes only TKIs that interact with ABC drug transporters

Mechanistic Insights into Interaction of TKIs with Target Kinases

Most of the TKIs that have been discussed above target the catalytic ATP-binding site of target kinases, thereby adopting a conformation almost identical to that used to bind ATP (the active conformation) (Manley et al. 2005). These TKIs work by inhibiting the accessibility of ATP to the active site of the kinase domain of target RTKs, thereby preventing phosphorylation of downstream targets, which in turn is essential for cellular proliferation (Manley et al. 2005). They were discovered using biochemical screens of highly active recombinant kinases, combined with structure–activity relationship (SAR) studies guided by medicinal chemistry approaches. The result was identification of the first and second generations of kinase inhibitors that preferentially bind to inactive conformations of the kinases (Liu and Gray 2006; Mol et al. 2004). Although the general mechanism of inhibiting ATP binding is similar for most TKIs, their selectivity and specificity comes from the orientation of binding to the ATP catalytic sites. For example, imatinib only binds to a specific inactive structure of the BCR-ABL kinase, while another potent BCR-ABL

inhibitor, PD173955, binds to this pocket in a less specific manner (Nagar et al. 2002). This information is derived from structures of the kinase domain of c-ABL in complex with PD173955 and imatinib (STI-571), suggesting that PD173955 can bind to multiple conformations of ABL (active or inactive), thereby achieving greater potency over imatinib (Nagar et al. 2002). Another example of structure-based specific inhibition of the BCR-ABL kinase is its inhibition by nilotinib, which is a phenylamino-pyrimidine derivative of imatinib (Weisberg et al. 2006). This drug was developed as a second-generation BCR-ABL kinase inhibitor for inhibiting imatinib-resistant kinases based on the crystal structure of the ABL-imatinib complex (Weisberg et al. 2005; Shah et al. 2004). Based on this structure, chemical changes were introduced to the imatinib molecule to obtain a more potent and selective BCR-ABL inhibitor. This resulted in the development of nilotinib, which is 10- to 30-fold more potent than imatinib in inhibiting the activity of the BCR-ABL tyrosine kinase (Kantarjian et al. 2006; Weisberg et al. 2005, 2006). Similar to imatinib, nilotinib binds to the inactive conformation of the ABL tyrosine kinase, with the P-loop folding over the ATP-binding site and the activation loop blocking the substrate-binding site, to disrupt the ATP-phosphate binding site and inhibit the enzyme's catalytic activity (Weisberg et al. 2005). This suggests that although the two-dimensional molecular structures of these two drugs might look similar, they are dissimilar in terms of spatial structure and molecular properties and thereby differ in their specificity and potency in interacting with the kinases. Dasatinib is another TKI that binds to the BCR-ABL kinase with less stringent conformational requirements than imatinib, and therefore exhibits increased potency but has reduced selectivity compared to imatinib (Tokarski et al. 2006). This drug also differs from most BCR-ABL kinase inhibitors in that it exclusively binds to the active conformation of the kinase (Tokarski et al. 2006). Taken together, these examples indicate that although mechanistically the TKIs are ATP-competitive inhibitors and their two-dimensional molecular structures in complex with these kinases look the same, the difference in functional groups really defines the specificity and their affinity for RTKs.

Mechanism of Substrate Binding and Efflux by ABC Drug Transporters

As stated earlier, ABC drug transporters recognize a wide array of amphipathic compounds including chemotherapeutics such as TKIs. Since efflux of these chemotherapeutics by ABC transporters results in decreased intracellular levels of drugs and the development of drug resistance, a major focus of research is to evaluate the mechanistic differences in the ability of P-gp or ABCG2 to recognize multiple, diverse substrates. Early studies probing into the mechanism of interaction of substrates with P-gp looked at the physicochemical properties of compounds that are substrates of P-gp and aimed to identify drug-binding sites and explain how

ATP hydrolysis is coupled with substrate transport (Ford et al. 1989). These studies in general reported that while physicochemical characteristics such as log*P* characteristics (Ford et al. 1989; Reis et al. 2013) and hydrogen-bond donor/acceptor properties (Seelig 1998a, b) are important for substrate recognition, aromatic moieties (Suzuki et al. 1997) and nitrogen atoms (Ford et al. 1989; Suzuki et al. 1997) within the drug-binding pocket are also essential for the modulation of the efflux properties of P-gp. Shapiro and Ling proposed that the major drug-binding site of P-gp contains two distinct drug-binding pockets that cooperate with each other (Shapiro and Ling 1997). They named them the H-site and the R-site, respectively. Identification of these sites with the ability to recognize multiple substrates provided a plausible explanation for the polyspecificity of P-gp by a "substrate-induced fit" model. In addition, specific residues in TMDs 4 (Ser222), 5 (Ile306), 6 (Leu339, Ala342), 10 (Ile868), 11 (Phe942, Thr945), and 12 (Gly984) were identified which were directly associated with substrate binding. Pleban et al. further defined the substrate-binding site of P-gp in each half of P-gp, next to TMDs 3/11 and 5/8 using a set of propafenone photoaffinity ligands in conjunction with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) (Pleban et al. 2005). Loo and Clarke, using site-directed mutagenesis in conjunction with disulfide cross-linking, demonstrated that the amino-acid residues facing the drug-binding pocket interact with functional groups of chemotherapeutics (Loo and Clarke 2008). Another study characterized the substrate-binding sites and efflux modulation mechanisms of P-gp using molecular docking studies using a refined model of mouse P-gp (Ferreira et al. 2013). We recently proposed that in addition to the structural flexibility of the drug-binding pocket that contains the primary and secondary drug-binding sites, molecular or chemical flexibility, which is dependent on the pharmacophoric features of ligands that bind to P-gp, also contributes to the binding of substrates to multiple sites, forming the basis of polyspecificity exhibited by P-gp (Chufan et al. 2013, 2015).

Pharmacophore modeling using quantitative structure–activity relationships (QSAR) is an approach that could be used to identify functional groups that may contribute to drug binding at the substrate-binding pocket. Several QSAR studies on a structurally homologous series of substrate compounds, such as verapamil, triazines, acridine carboxamides, phenothiazines, thioxanthenes, flavones, dihydropyridines, propafenones, and cyclosporine A derivatives, have been reported (Ecker et al. 2008). Pharmacophore modeling was also used to characterize properties of an ideal substrate that are needed for spatial rearrangement of substrates in the drug-binding pocket of P-gp and ABCG2 (Pearce et al. 1989; Ferreira et al. 2011; Pajeva et al. 2009). Demel and colleagues suggested that features such as charge, hydrogen-bond acceptors, and hydrophobicity commonly influence substrate–transporter interaction and these features are considered to be a common pharmacophoric theme that may explain the overlapping substrate specificity of ABC transporters (Demel et al. 2008). In another report, Cramer et al. studied propafenone analogs for their interaction with P-gp and ABCG2 and defined distinctly different feature-based interactions such as charge, hydrogen bonding, and hydrophobicity (Cramer et al. 2007).

Recent progress in identifying structural aspects of drug binding to P-gp has come from solving the crystal structure of P-gp from the murine and *C. elegans* species (Ward et al. 2013; Li et al. 2014; Aller et al. 2009; Jin et al. 2012). The crystal structures of mouse P-gp in complex with synthesized cyclic peptides, QZ59-RRR and QZ59-SSS, presumed to occupy the substrate-binding pocket of the transporter, showed a large internal drug-binding pocket of approximately 6000 $Å^3$, which was open to the intracellular compartment and the lipid membrane through two 'gates' formed by TMD pairs 4/6 and 10/12.

All of the above studies suggest a large drug-binding pocket within the internal cavity of P-gp but the precise mechanism of these substrate–transporter interactions as it relates to the amino-acid composition of drug-binding pockets and physicochemical characteristics of substrates still remains largely unknown. These studies have made incremental but significant contributions to characterize the general substrate-binding properties of ABC drug transporters and have identified amino-acid residues that line the drug-binding pocket in these transporters. However, none of these reports discussed the binding site for TKIs or provided information about common pharmacophoric features that may be essential for interaction of TKIs with the drug-binding pocket of ABC transporters.

Structure-Based Prediction of TKI Interaction with ABC Drug Transporters

With the availability of several crystal structures of TKIs bound to their target kinases, mechanistic details of the binding have been elucidated at the atomic level (Table 2). However, details about their interactions with transporters are still poorly understood. A significant step in this direction would be to obtain the crystal structure of either P-gp or ABCG2 bound to these TKIs, which is not available yet. In the absence of the crystal structure of this complex, identification of the functional groups or key chemical moieties of TKIs and the amino-acid residues in the drug-binding pocket of the transporter that interact with the TKIs would provide more information on the mechanism of interaction of these drugs as substrates or inhibitors of ABC drug transporters.

We have studied multiple TKIs and have biochemically characterized the mechanism of interaction of these TKIs with ABC drug transporters. It is clear that although these TKIs were originally developed to competitively inhibit ATP binding to the kinases, they do not interact at the ATP-binding pocket of ABC transporters (Shukla et al. 2008a, b, 2012). For imatinib and nilotinib, we specifically showed that these two TKIs do not compete for the binding of ATP to the NBDs of P-gp or ABCG2. Instead, they behave as typical substrates/modulators of these transporters, as they stimulate ATPase activity and the vanadate-induced trapping of ADP after hydrolysis at the NBDs by occupying the substrate-binding pocket in the transmembrane domains of the transporters (Shukla et al. 2008a, 2012).

Developing a pharmacophore model using three-dimensional QSAR can help to identify key chemical features that are required for specific ligand/receptor interactions. As described above, several pharmacophoric features were detailed in the general context of substrate interactions with ABC drug transporters but specific features of any TKIs necessary for binding to ABC transporters have not yet been elucidated. Therefore, we will now discuss our work on the interaction of imatinib and nilotinib at the substrate-binding pockets of human P-gp and ABCG2.

We studied the pharmacophoric features of nilotinib to understand its distinctive interactions with both P-gp and ABCG2 and to determine if these features were different than those required for inhibiting BCR-ABL kinase activity (Shukla et al. 2014). Structural derivatives of nilotinib were used to study their inhibitory activity toward P-gp, ABCG2, and BCR-ABL kinase. The pharmacophore identified includes structural features such as hydrophobic groups, aromatic rings, and hydrogen-bond acceptor/donor groups that contribute to nilotinib's interaction with P-gp at the substrate-binding pocket (Shukla et al. 2014). Moreover, significant overlap of pharmacophoric features was observed for its inhibitory activity not only toward P-gp and ABCG2, but also towards BCR-ABL kinase. Although the data set was derived from 25 compounds, it could be expanded with more derivatives to validate and construct a better model that may be able to predict the ability of nilotinib to inhibit various ABC transporters. Tiwari et al. also described the interaction of nilotinib with P-gp and ABCG2 as a class effect of phenylpyrimidine-derived BCR-ABL TKIs, which could be more general in nature. Other drugs belonging to a similar class with these structural features would be expected to interact with the transporters (Tiwari et al. 2013).

We used molecular docking complemented with mutational analysis and a structural modification approach to identify nilotinib's binding pocket in P-gp (Shukla et al. 2014). The docked orientation of nilotinib was not only validated by site-directed mutagenesis of selected residues but also by using structural derivatives of nilotinib. The data suggest that while the pyridine and pyrimidine moieties of nilotinib are important for interaction at the drug-binding pocket of P-gp, the imidazole group is not critical for this interaction (Fig. 3). Moreover, we identified three main residues in the drug-binding pocket of P-gp (Y307, M949, and A985) that interact with three major functional groups (pyridine, CF_3, and imidazole) of nilotinib (Fig. 4). The interaction of nilotinib at this pocket was validated using biochemical studies with mutants that provided experimental support to the in silico docking studies. This study identified the primary site of nilotinib binding on P-gp, with Y307 interacting with the pyridine ring, A985 interacting with the trifluoromethylphenyl group, and M949 interacting with the imidazole ring of nilotinib (Fig. 4). In addition, comparison of the docked model of imatinib and nilotinib indicated that the piperazinylmethylphenyl moiety in imatinib is oriented differently than the imidazole moiety in nilotinib in this binding pocket (Fig. 4) (Shukla et al. 2014). The model also explained the observed differences in affinities between

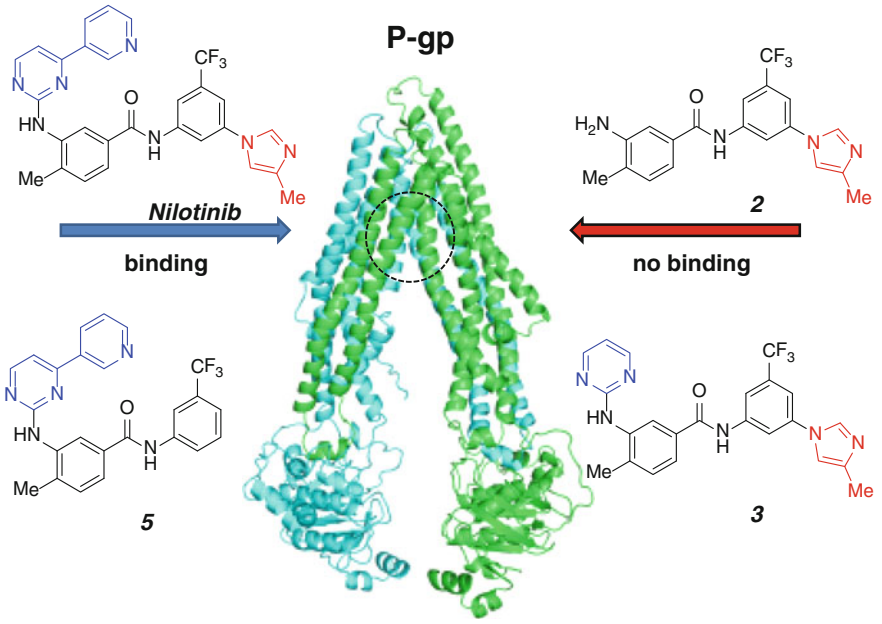

Fig. 3 Nilotinib's binding site on human P-gp. The drug-binding pocket in the homology model of human P-gp is indicated by a *black circle*. Schematic based on SAR and directed mutational studies shows that pyridine and pyrimidine moieties (*blue*) in the nilotinib structure are required for interaction of nilotinib at the drug-binding pocket of P-gp, whereas the imidazole moiety (*red*) is not essential for its interaction with the transporter. The structures of nilotinib and its derivatives #2, 3, and 5 are shown [based on data in Shukla et al. (2014)]

imatinib and nilotinib for their inhibitory activity toward P-gp, as the piperazinylmethylphenyl moiety of imatinib was bound in an orientation that was different for nilotinib. The docking analysis complemented with SAR and mutational analysis corroborated well with the binding differences to the target Abl kinase that was elucidated through the crystallographic structures of nilotinib (PDB ID: 3CS9) (Weisberg et al. 2005) and imatinib (PDB ID: 2HYY) (Cowan-Jacob et al. 2007) with Abl kinase. Collectively, the binding orientations of nilotinib and imatinib in nilotinib-Abl and imatinib-Abl appeared similar to nilotinib-P-gp and imatinib-P-gp complexes, suggesting that the models of docking of imatinib and nilotinib on P-gp provided a rationalized view of the experimentally observed affinity differences between imatinib and nilotinib for binding to P-gp. To our knowledge, this is the only report that specifically identified the primary binding site of nilotinib in the drug-binding pocket of P-gp and the structural features that are important for interaction with P-gp. We proposed that the next generation of TKIs should be developed with alternative structural features that would not allow the inhibitors to be recognized by P-gp or other ABC drug transporters.

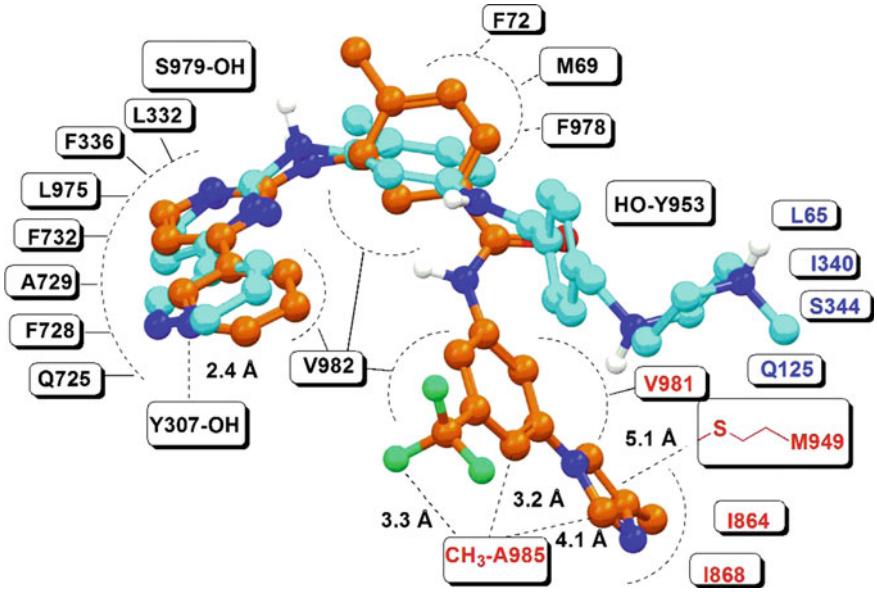

Fig. 4 Differences in binding orientation of nilotinib and imatinib docked in the drug-binding pocket of human P-gp. Important amino acids in the drug-binding pocket are depicted schematically along with nilotinib (*orange*) and imatinib (*cyan*) as ball-and-stick models. The amino-acid residues that interact with both imatinib and nilotinib, with only imatinib, and with only nilotinib are shown in *black*, *blue*, and *red* colors, respectively (reproduced from Shukla et al. (2014))

Conclusions and Perspective

Preclinical and clinical studies collectively demonstrate that the interaction of TKIs with ABC transporters influences the pharmacokinetics and pharmacodynamics of these drugs as well as co-administered drugs in combination therapy, leading to drug–drug interactions. Moreover, dietary components from food and food supplements can modulate the function of transporters and therefore have the potential to alter the effectiveness of TKI therapy. While in this review we have specifically discussed TKI interactions with ABC transporters, the uptake transporters, organic anion-transporting polypeptides (OATPs) and organic cation transporters (solute carrier transporters (OCTs (SLCs)), which are associated with drug uptake in the intestines and drug detoxification in hepatocytes, have also been shown to interfere with TKI pharmacokinetics (Polli et al. 2008; Reyner et al. 2013; Yamakawa et al. 2011). These findings suggest that it is important to understand the mechanistic interactions of TKIs with these transporter proteins. In the absence of crystal structures of ABC transporters bound to TKIs, the strategies described above such as molecular docking, mutational mapping, and SAR, extensively used for studying the mechanism of drug/substrate interaction with ABC transporters, must be used to

measure the binding properties of TKIs with ABC drug transporters. These structural details will help to identify the TKI-binding site in the drug-binding pocket of the transporters. Specifically, similar to what is known about interaction of TKIs with target kinases, identification of key structural features and characterization of their binding pockets on ABC transporters will help in designing novel scaffolds that are ideal TKIs. The SAR, modeling, and mutational studies complemented with in vitro and in vivo studies can therefore be used to develop ideal TKIs. The ideal kinase inhibitor would be one that exhibits minimal interaction with ABC (efflux) or uptake (influx) transporters. As the number of kinase inhibitors entering clinical trials increases rapidly, their clinical efficacy and potency will depend on their off-target interactions not only with ABC drug efflux pumps, but also with uptake transporters (not discussed here). Understanding the mechanistic basis for their interaction with transporters will help in developing novel TKIs, with a greater degree of selectivity toward kinases and better clinical efficacy. We believe the administration of one or more TKIs with standard chemotherapy regimens will help to eliminate cancer cells more effectively.

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Biology of Mitochondrial ABCs and Their Contribution to Pathology

Yu Fukuda, John A. Lynch and John D. Schuetz

Abstract The mitochondria are key determinants in the life and death of a cell, and abnormalities in mitochondrial function are associated with a number of pathological conditions. Given that many crucial co-factors are generated in mitochondria, including heme and iron–sulfur clusters, it is a reasonable expectation that energy-dependent transporters might facilitate the efficient movement of these molecules through membrane barriers to their next enzymatic steps. The four mitochondrial ABC transporters are ABCB6, ABCB7, ABCB8, and ABCB10. Of these, ABCB6, ABCB7, and ABCB10 have been implicated in processes important to erythroid cells. Mitochondria are also the site of energy production, during which cytotoxic reactive oxygen species are generated. Through modulation of iron and heme biosynthesis, all four mitochondrial ABC transporters have important roles in protecting cells against excessive ROS. In humans, loss-of-function mutations in ABCB7 cause X-linked sideroblastic anemia with ataxia, and several variants that result in loss of ABCB6 at the surface of red cells have been used to identify a new blood group, *Lan*. ABCB8 has an important role in ischemia. Two of the mammalian mitochondrial transporters, ABCB6 and ABCB7, are closely related to homologs which serve to protect lower organisms from heavy metal toxicity, and mammalian ABCB6 has been demonstrated to provide resistance against some toxic metals, though the mechanism remains unclear. The physiology of mitochondrial transporters and how they contribute to pathological states will be discussed.

Keywords Mitochondria · Heme · Iron · Heavy metal · Reactive oxygen species · ABC transporter

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Abbreviations

ABC	ATP-Binding cassette
ALA	δ-aminolevulinic acid
ANT	Adenine nucleotide translocator
ATPase	decompose ATP
Fe–S	iron–sulfur
HMT1	Heavy metal tolerance protein
mitoK$_{ATP}$	Mitochondrial ATP-sensitive K^+
MDS	Myelodysplastic syndrome
NBD	Nucleotide-binding domain
PIC	Phosphate carrier
PPIX	Protoporphyrin IX
RARS	Refractory anemia with ring sideroblasts
ROS	Reactive oxygen species
SDH	Succinate dehydrogenase
SUR	Sulfonylurea receptor
XLSA/A	X-linked sideroblastic anemia with cerebellar ataxia

Introduction

The mitochondria are key regulators in cellular processes from the execution of apoptosis to generation of energy. The electron transport chain generates energy necessary for the cell, but an inherent by-product is the production of reactive oxygen species (ROS), which can damage proteins, lipids, and DNA. Many steps in the biosynthesis of essential molecules, such as heme, iron–sulfur (Fe–S) clusters, urea, steroid, and neurotransmitters, take place in the mitochondria. In addition, mitochondria are involved in the storage and homeostasis of iron. Mitochondrial iron is essential for the formation of heme and Fe–S clusters, which are co-factors to many enzymes with crucial roles in cellular events including oxidative phosphorylation.

As the major site of cellular metabolism, defects in mitochondrial proteins result in a variety of pathological conditions. For example, porphyrias reflect aberrant heme biosynthesis, whereby elevated levels of intermediates in heme metabolism (porphyrins) cause intracellular damage (Balwani and Desnick 2012). As hemoproteins are involved in protection against oxidative stress, disrupted heme biosynthesis produces oxidative damage. Some intermediates of defective heme synthesis are capable of forming reactive superoxides which account for their cytotoxicity. Efficient mitochondrial heme production also needs coordination with an iron delivery process, as excess iron results in oxidative stress. Impaired assembly of Fe–S clusters is inextricably linked to increased free iron in mitochondria. Thus, perturbations in iron homeostasis, heme biosynthesis, and Fe–S cluster formation are likely to produce oxidative stress.

Table 1 Summary of human mitochondrial ABC transporters

	Ortholog	Chr. location	Mitochondrial targeting sequence	Mito. location	Role	Substrates	Disease or human phenotype	Knockout	Protection against oxidative stress	Expression during erythroid differentiation
ABCB6	Hmt1	2q36	None N-terminal MSD_0 contains *N*-glycosylation and disulfide bond	OM	Heme biosynthesis	Porphyrins Binding and transport tested	Lan	Viable-elevated porphyrin biosynthesis Increased sensitivity to Phz toxicity	Increases hemoproteins such as catalase	Increase
ABCB7	Atm1	Xq12–q13	1–53	IM	Fe–S biogenesis	Sulfur-containing molecules GSH-conjugated and Fe–S-structure and ATPase in yeast	XLSA/A RARS	Embryonic lethal Mx1-Cre shows sideroblasts	Iron, Fe/S homeostatis	Increase
ABCB8	(Mdl1?)	7q35–36	1–55	IM[a]	Iron export (indirect)?	ND		Cardiac-specific knockout mild cardiomyopathy	Through reduction in mito iron	
ABCB10	Mdl1	I1q32	1–105	IM	Heme biosynthesis	ND ALA?		Embryonic lethal Mx1-Cre-anemia and iron accumulation	MitoROS, hemoproteins	Increase

[a]Not formally tested

There are four ATP-binding cassette (ABC) 'half-transporters' (one membrane-spanning domain and nucleotide-binding domain (NBD) in the gene) from the B subfamily of the ABC protein superfamily that have been shown to localize in mitochondria (Table 1). ABCB7, B8, and B10 localize to the mitochondrial inner membrane (Ardehali et al. 2004; Csere et al. 1998; Graf et al. 2004; Hogue et al. 1999; Taketani et al. 2003), whereas ABCB6 localizes to the outer membrane and most recently at the plasma membrane (Helias et al. 2012; Krishnamurthy et al. 2006; Mitsuhashi et al. 2000; Paterson et al. 2007). However, in this review, we will focus on the role of ABCB6 in mitochondria as little is known about its function at the plasma membrane. In the past few years, knockout animal models were generated for each of these mitochondrial ABC transporters, providing insights into their role in mitochondrial function. However, endogenous ligands have yet to be identified for ABCB8 and ABCB10. Nonetheless, available data (discussed below) suggest that all four mitochondrial ABC transporters at least contribute to iron and heme homeostasis (Fig. 1). This might account for why oxidative stress occurs in their absence. Interestingly, ABCB6, ABCB7, and ABCB10 all are highly expressed in erythroid progenitors, suggesting a prominent role in erythroid biology (Fig. 2), and their expression levels increase during erythroid differentiation (Taketani et al. 2003).

Exposure to xenobiotic agents is an ever-present threat to life. Heavy metals are an important subclass of these and organisms have adopted a wide range of strategies to cope with their presence in the environment. In plants, these mechanisms range from chelation by small molecules to incorporation into vacuoles (Hall 2002). In yeast and mammals, some heavy metal detoxification occurs through the excretion of glutathione-bound conjugates by plasma membrane ABC transporters

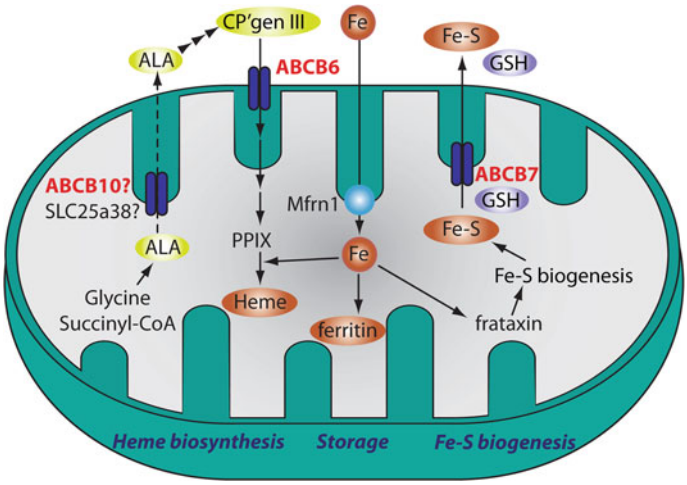

Fig. 1 ABC transporters and iron utilization in mitochondria. *ALA* δ-aminolevulinic acid; *CP'gen III* coproporphyrinogen III; *PPIX* protoporphyrin IX; *GSH* glutathione; *Mfrn1* mitoferrin

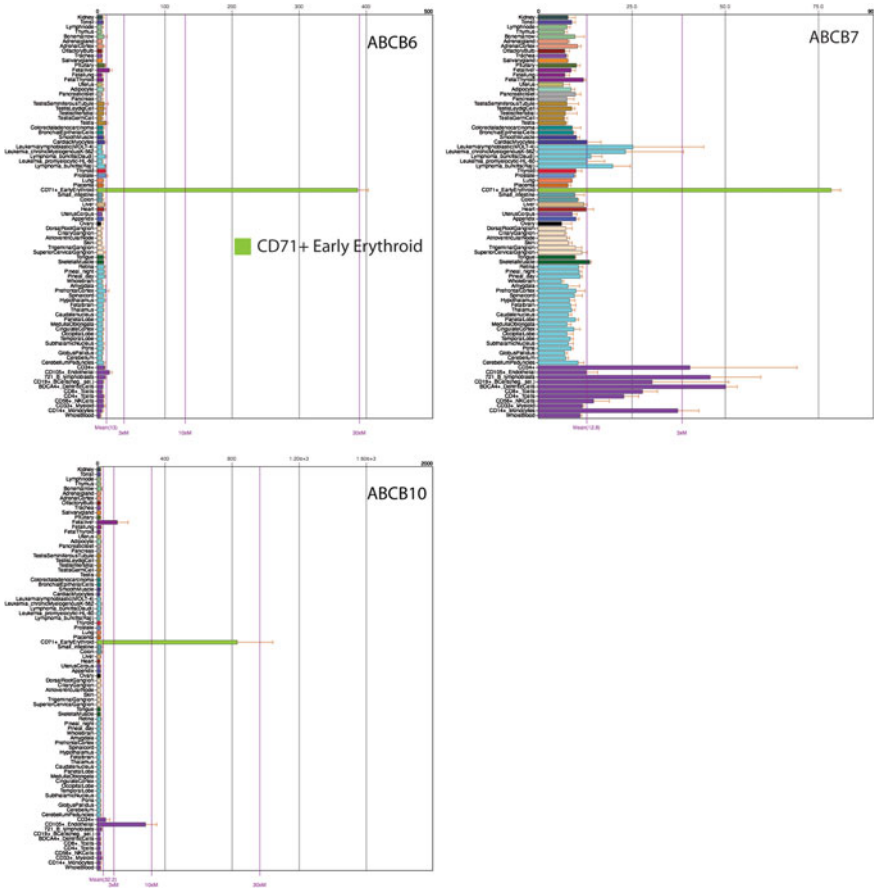

Fig. 2 *ABCB6, B7*, and *B10* are expressed at high levels in CD71+ early erythroid progenitors. Data obtained from biogps.org

(e.g., ABCC1, ABCC4) (Leslie et al. 2004). In yeast, ABC transporters ycf1p and Bpt1b reside in vacuolar membranes where they import and sequester heavy metals. Recent evidence demonstrates that some ABCB mitochondrial transporters are capable of providing resistance to heavy metals (Preveral et al. 2009).

Heavy Metal Toxicity and HMT1 Homologs

While many heavy metals, such as zinc, are necessary for metabolic processes such as reduction of alcohols by alcohol dehydrogenase, dissipation of free radicals by superoxide dismutase (Gautam et al. 2012), and the synthesis of methionine

(Jing et al. 2014), others, such as arsenic and cadmium, are harmful, and environmental exposure disrupts metabolic processes. Among the mechanisms organisms have developed for defense against cadmium toxicity is the sequestration of the chelated ion, a process involving the heavy metal tolerance proteins (HMT1) (Preveral et al. 2009). One protective mechanism that was characterized in the yeast strain, *Schizosaccharomyces pombe*, showed that the ABC half-transporter, *Sp*HMT1, was necessary for cadmium detoxification (Ortiz et al. 1992). Specifically, *Sp*HMT1 imported cadmium complexed with metal-binding peptides, the phytochelatins, into vacuoles. In higher animals, *Sp*HMT1 is homologous to the mammalian mitochondrial transporters, ABCB6 and ABCB7, and *Ce*HMT in *Caenorhabditis elegans*. *Sp*HMT1, for example, reduces cadmium toxicity in *Saccharomyces pombe* and *Caenorhabditis elegans*, *Ce*HMT1 performs likewise, indicating a conserved function (Schwartz et al. 2010). One homolog of *Sp*HMT1, *Na*Atm1, from *Novosphingobium aromaticivorans*, has been crystallized with the tripeptide glutathione, which provides the structural basis for detoxification related to glutathione binding and transport (Lee et al. 2014). A binding site for glutathione and its derivatives in *Na*Atm1 was demonstrated, as well as a capacity to confer heavy metal resistance, a function which is lost when a critical residue in the Walker B motif in the NBD is mutated.

Arsenic remains one of the most common toxic heavy metals humans are exposed to and recent reports implicate ABCB6 as a protector against arsenic toxicity. Given its evolutionary relationship with *C. elegans* and *S. pombe* orthologs, this is not unexpected. Reports indicate arsenate exposure increases ABCB6 expression in vitro and in vivo (Chavan et al. 2011). These authors further demonstrated that ABCB6 knockdown in human liver cancer cell lines, HepG2 and Hep3B cells, was detrimental to survival in media containing arsenic. Together, the findings of heavy metal resistance by HMT1-related proteins suggest a conserved function.

The mechanism by which ABCB6 provides resistance to arsenic, or other heavy metals, in higher organisms is not immediately obvious. In fact, the localization of ABCB6 to the mitochondria provides a challenge in explaining the mechanism of resistance, as its outer mitochondrial membrane localization and orientation would lead to direct import of arsenic into the mitochondria. As mitochondria are key targets for heavy metal toxicity (Belyaeva et al. 2008), a new mechanism is required to account for this. Clearly, in contrast with the yeast vacuoles that use HMT1 proteins to sequester heavy metals, import of heavy metals by ABCB6 into the mitochondria is unlikely to produce any salutary effect. In fact, one would suspect heavy metals in mitochondria might be deleterious. One proposition is that ABCB6 is a functional paralog of *Na*ATM and co-transports glutathione. This might counteract the ROS produced by heavy metal disruption of the electron transport chain (Fig. 3). However, it is difficult to imagine the survival advantage of mitochondrial import of glutathione co-transport with a heavy metal.

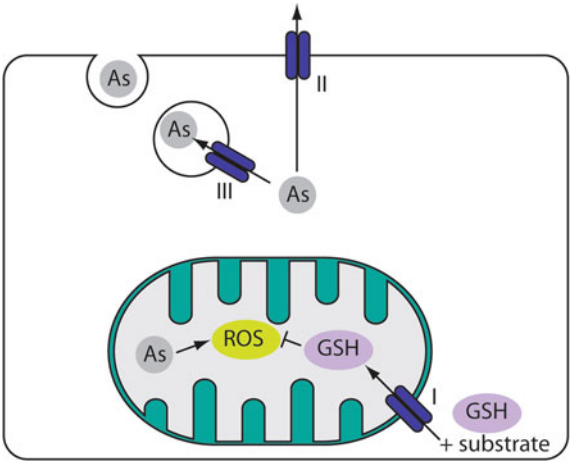

Fig. 3 Detoxification of heavy metals by ABC transporters. ATM1 analogs, such as ABCB6, could mitigate cellular damage (I) by importing GSH with other substrates, which could reduce the damage inflicted by heavy metal ROS generation within the mitochondria, (II) by directly exporting heavy metals out of the cell, or (III) by compartmentalizing metals into an unknown structure where they might be secreted from the cell. *As* arsenic/heavy metal; *ROS* reactive oxygen species; *GSH* glutathione

Artesunate

Mitochondrial transporters have also been implicated in resistance to chemotherapeutic agents that affect heme. For example, ABCB6 expression is upregulated by exposure to artesunate (Kelter et al. 2007). Artesunate is one of the modern classes of drugs used in the treatment of malaria, caused by the microbe *Plasmodium falciparum* (Dondorp et al. 2005). A member of the artemisinin class of compounds, artesunate's mechanism of action involves the generation of free radicals secondary to its reaction with the iron of heme (Pandey et al. 1999). Recently, artesunate has shown promise as a cancer chemotherapeutic, where its effectiveness for some tumors is also dependent upon the tumor's iron or heme concentration (Hamacher-Brady et al. 2011). Again, this mechanism also relies upon levels of sufficient heme production by the target tissue. This suggests that mitochondrial ABC transporters involved in heme and iron homeostasis might have a significant role in cellular response to this drug. Given ABCB6 role in heme generation in vitro and in vivo, one might suspect ABCB6 deficiency could impact responses to artesunate (Ulrich et al. 2012).

ABCB6

Background

ABCB6 was initially cloned and named MTABC3. It localized to chromosome 2q36 and was homologous to ATM1P, a *Saccaromyces cerevisiae* protein that regulates iron–sulfur clusters egress from the mitochondria to the cytosol (Mitsuhashi et al. 2000). The functional ortholog of ATM1P was eventually determined to be ABCB7. Nonetheless, the overexpression of ABCB6 was capable of partially overcoming the inherent iron accumulation defect in ATM1P deficient yeast, suggesting its mitochondrial localization contributed to mitochondrial iron homeostasis. Further characterization of ABCB6 indicated that it formed a homodimer at the outer mitochondrial membrane and it accelerated heme biosynthesis (Krishnamurthy et al. 2006). While both its regulation by heme and its high degree of expression, in early erythroid and liver cells, suggested an important role in heme regulation within the mitochondria, the lack of any known mitochondrial targeting sequence presents difficulties in explaining this localization (Tsuchida et al. 2008). Later, subcellular fractionation indicated that ABCB6 was localized to two locations within the cell, one mitochondrial and the other the plasma membrane (Paterson et al. 2007). Other locations have since been suggested, including lysosomes (Kiss et al. 2012) and Golgi (Tsuchida et al. 2008), but one could argue these locations are secondary and are related to either processes involved in ABCB6 biosynthesis and/or degradation. Recent studies have identified motifs in ABCB6 that are important for trafficking, such as the requirement of a pair of conserved cysteines that are required for ABCB6 stability and to exit from the ER (Fukuda et al. 2011). Most recently, a transmembrane domain has been identified as important for the movement of ABCB6 from plasma membranes into rab5-containing vesicles, which suggests it undergoes endosomal retrieval (Kiss et al. 2015). Undoubtedly, future studies will determine how ABCB6 traffics to the mitochondria and the plasma membrane.

ABCB6 and Porphyrin Transport

ABCB6 has a bona fide role in porphyrin transport. The movement of porphyrins, such as heme and protoporphyrin IX (PPIX), through membrane barriers via transport, was thought to occur by passive diffusion. We now know of a wide range of carriers and transporters for porphyrins, including those present in mammals with a specificity for heme, such as HCP1 (SLC46A1) (Le Blanc et al. 2012), FLVCR (slc49A1/2) (Keel et al. 2008), and ABCG2 (Gnana-Prakasam et al. 2011; Khan and Quigley 2011), which reportedly also localizes to mitochondria, although we do not know exactly where it localizes (e.g., inner membrane, outer membrane)

(Kobuchi et al. 2012). In erythroid cells, diffusion would be an inefficient mechanism to control porphyrin movement into the mitochondria and would hamper hemoglobin production during erythroid differentiation. An active transporter, which could aid in this process, could allow for high rates of heme required to synthesize hemoglobin. Available evidence indicates ABCB6 is the only ATP-dependent porphyrin transporter capable of importing porphyrins into the mitochondria (Chavan et al. 2011; Lynch et al. 2009; Ulrich et al. 2012). The ATP-dependent transport of porphyrins into the mitochondria, particularly the heme precursor copropophyrinogen III, has long been established (Ponka 1999; Rebeiz et al. 1996), but the molecular entity that performed this was unknown. ABCB6, as an outer mitochondrial transporter, with its nucleotide-binding domains facing the cytosol and levels regulated by porphyrin concentration, was a strong candidate. Further, binding studies demonstrated that ABCB6 has a preference for binding tetrapyrroles like heme and not monopyrroles. Transport studies using ^{55}Fe-labeled hemin showed that it could be imported into mitochondria in an ATP- and ABCB6-dependent manner (Krishnamurthy et al. 2006). It is not known if the cytosolic coproporphyrinogen III is actively transported by ABCB6 (Ulrich et al. 2012). This is because the rapid oxidation of copropophyrinogen III to coproporphyrin III precludes its ready use as a transport substrate in vitro. However, coproporphyin III retains the primary tetrapyrrole feature of coproporphyrinogen III (differing only by two double bonds), and was shown to be readily transported by ABCB6 in mouse liver mitochondria (Ulrich et al. 2012). Interestingly, a passive coproporphyrin uptake transport component existed regardless of ABCB6 presence.

Porphyrin and Physiological Significance

As would be expected as a consequence of porphyrin transport, ABCB6 has been proposed as a regulator of heme synthesis. The overexpression of ABCB6 was shown to enhance the cellular levels of the penultimate heme precursor, PPIX, in cultured K562 human erythroblasts (Krishnamurthy et al. 2006). Studies using either a non-functional ABCB6 (harboring a mutation in the Walker A domain) or a mutation in a crucial cysteine (required for stability) exhibited reduced formation of PPIX (Fukuda et al. 2011). This increased porphyrin generation by ABCB6 was further enhanced by the co-addition of δ-aminolevulinic acid (ALA), an early porphyrin precursor, whose presence enhances the concentration of porphyrin biosynthetic intermediates. Curiously, the lack of ABCB6, demonstrated in *Abcb6*$^{-/-}$ mice, was associated with an increase in PPIX in reticulocytes (Ulrich et al. 2012). This unexpected result is related to compensatory adaptations where deficiency of Abcb6 drives changes in the expression of genes involved in both heme biosynthesis and degradation.

ABCB6 and Disease

Evidence suggests that the function of ABCB6 may be an important prognostic factor in some cancers. This is demonstrated by its capacity to enhance the accumulation of PPIX in human gliomas (Zhao et al. 2013). These authors found a strong correlation between ABCB6 expression and prognostic characteristics of the tumor, such as higher amounts of ABCB6 in those with both a higher grade and more aggressive glioma. In addition, the amount of PPIX generated by treatment with a porphyrin biosynthetic precursor, ALA, was greater in those expressing more ABCB6. These findings are in agreement with ABCB6 as a positive regulator of porphyrin biosynthesis. However, it is unknown exactly how an increase in porphyrin biosynthesis might contribute to a more aggressive tumor phenotype.

ABCB6 role in synchronizing the coupling between heme biosynthesis and hemoglobin production was demonstrated in *Abcb6*-null animals (Ulrich et al. 2012). Absence of Abcb6 in mice and humans does not produce any obvious pathological condition under normal unperturbed circumstances (Helias et al. 2012). However, in an experimental condition mimicking profound anemia, Abcb6 was essential for normal stress erythropoiesis. In this work, mice exhibited adaptive changes which, coupled with the stress of enhanced porphyrin generation but decreased heme formation, produced a profound defect in *Abcb6* knockout mice survival (Ulrich et al. 2012).

Clearly, ABCB6 role in porphyrin homeostasis is important in pathological conditions. Some roles are clear, like the coupling accelerated heme biosynthesis to hemoglobin production while others are less so. For example, how does Abcb6 promote resistance to heavy metals? Does ABCB6 expression change the biology of gliomas? Based upon ABCB6 impact on some enzymes (e.g., the mitochondrial electron transport chain (Lynch et al. 2009), it is likely that ABCB6 importance extends to other areas as well. Many unanswered questions remain to be addressed. For example, without a canonical targeting sequence, how does ABCB6 localize to the mitochondria? What controls its differential localization between cellular compartments like mitochondria and plasma membrane? Does the existence of a loop formed by conserved cysteine signify any importance of redox in its trafficking? Presumably, all of these questions will be soon answerable given the current interest in ABCB6.

ABCB7

Background

ABCB7 was first identified as the causative gene for a form of X-linked sideroblastic anemia with cerebellar ataxia (XLSA/A). Further, ABCB7 has been implicated in another form of anemia, refractory anemia with ring sideroblasts (RARS).

The human *ABCB7* sequence was identified in the expressed sequence tag (EST) database based upon its sequence homology with yeast *Atm1* (discussed below) (Shimada et al. 1998), and mapped to chromosome Xq12–13 (Savary et al. 1997). Human *ABCB7* shares ~50 % sequence identity with yeast *Atm1* and is highly conserved in other species. Most of the functional studies on ABCB7 have come from the use of an *Atm1*-insufficient yeast strain, in which ABCB7 expression rescues both the aberrant mitochondrial iron accumulation and lack of Fe–S cluster proteins, suggesting a conserved function (Csere et al. 1998). ABCB7 is predicted to have six membrane-spanning helices with a C-terminal NBD. Mitochondrial localization of ABCB7 was confirmed by subcellular fractionation as well as immunofluorescence microscopy (Csere et al. 1998; Taketani et al. 2003). Unlike ABCB6, ABCB7 harbors an N-terminal mitochondrial targeting sequence in the first 135 amino acid residues that is capable of targeting a cytosolic protein, dihydrofolate reductase, to the mitochondria (Csere et al. 1998).

Function Inferred from ABCB7 Homolog Atm1p

The first mitochondrial ABC transporter identified, appropriately named Atm1 for ABC transporter of mitochondria, was essential for normal growth, iron homeostasis, and formation of Fe–S clusters in yeast (Kispal et al. 1997, 1999; Leighton and Schatz 1995; Senbongi et al. 1999). Immunofluorescence microscopy and subcellular fractionation demonstrated mitochondrial localization, consistent with its 53 amino acid targeting sequence being sufficient to target an unrelated protein to the mitochondria. Atm1p orientation and localization in the mitochondria was determined by the proteinase K insensitivity of a C-terminally-tagged Atm1p both in purified mitochondria and mitoplasts. These studies indicated Atm1p resides in the inner mitochondrial membrane with the C-terminal NBD facing the matrix. From this orientation, Atm1p was proposed to transport its substrates from the matrix into the intermembrane space. The reduction in Fe–S cluster-containing proteins and holo-forms of hemoproteins in a yeast strain lacking *Atm1* suggested its substrates were Fe–S clusters (Kispal et al. 1997; Leighton and Schatz 1995). Coupled with these deficiencies was enhanced mitochondrial iron accumulation which, undoubtedly, accounted for the increased oxidative stress observed in Δ*atm1* cells. As might be expected, Δ*atm1* cells were hypersensitive to oxidative stress and exhibited impaired growth in the presence of H_2O_2 (Kispal et al. 1997; Senbongi et al. 1999). Further evidence of functional conservation was suggested by *C. elegans* lacking the homolog of *ABCB7*, *abtm-1*; free iron and oxidative stress increased in this strain (Gonzalez-Cabo et al. 2011).

While Atm1 appears integral to providing cytosolic Fe–S clusters to certain apo-proteins, its direct role in Fe–S cluster transport has not been confirmed. In eukaryotes, the multi-step Fe–S cluster assembly occurs in the mitochondrial matrix where the cysteine desulfurase, Nfs1p, provides elemental sulfur and frataxin acts as an iron chaperone. Lack of *Nsf1* (in yeast) results in a complete deficiency in

both mitochondrial and cytosolic Fe–S-containing proteins. In contrast, *Atm1* absence only affects cytosolic Fe–S-containing proteins (Kispal et al. 1999). From this, one infers that Atm1 is primarily involved in Fe–S cluster transport, but not in their biogenesis.

Fe–S-containing proteins are involved in multiple cellular processes such as electron transport and heme biosynthesis. Fe–S cluster proteins such as iron regulatory protein 1 (IRP1, holo-protein acts as c-aconitase) use Fe–S cluster to sense cellular iron levels and ultimately regulate the expression of proteins involved in iron homeostasis. In mammals, ferrochelatase, the enzyme responsible for the insertion of iron into PPIX to make heme, requires an Fe–S cluster inserted into its C-terminus (Crooks et al. 2010). In total, mitochondrial iron, Fe–S cluster, and heme biosynthesis share intimate regulatory overlap.

The homodimeric crystal structure of Atm1 revealed a binding site for glutathione (GSH), suggesting thiol-containing compounds are endogenous substrates (Lee et al. 2014; Srinivasan et al. 2014). Both *Saccharomyces cerevisiae* Atm1 and bacterial *Na*Atm1 ($\sim$45 % sequence identity with yeast Atm1 and human ABCB7) were crystallized in a nucleotide-free state, with structures solved in either the GSH- or GSSG-bound (oxidized GSH) states, respectively (Lee et al. 2014; Srinivasan et al. 2014). In the absence of nucleotides, Atm1 adopted an inward-facing open conformation with GSH binding at positively charged residues in an internal cavity. Because the structure was similar to the GSH-free form, it suggested some substrates bind without producing conformational changes. Nucleotide binding to the NBDs on a substrate-bound ABC transporter induced a conformational change to an outward-facing conformation with substrates released to the other side. Overall, these Atm1 structures showed good agreement with previously reported ABC transporters, i.e., where transmembrane helices from each monomer intertwined with the helices from the other monomer. Notably, the hydrophilic amino acid residues where GSH binds are conserved in human ABCB7.

Several GSH and related compounds stimulated ATPase activity of *Na*Atm1, which suggests that these compounds are ligands for this transporter, with the highest activity observed for metallated, aromatic hydrocarbon-conjugated, and oxidized GSH-related compounds (Lee et al. 2014). For example, S–Ag GSH and S–Hg GSH strongly activated *Na*Atm1 ATPase activity. Furthermore, overexpression of *Na*Atm1 in metal-sensitive *E. coli* strains protected against toxic amounts of heavy metals. *Na*Atm1 also mediated ATP-dependent transport of GSSG into the vesicles prepared from *E. coli* membranes. These results suggested that Atm1 alleviates heavy metal toxicity through export of metallated GSH. Because GSH is involved in extra-mitochondrial Fe–S cluster assembly, GSH may be a part of the substrate (e.g. GSH-coordinated Fe–S clusters).

A further benefit of the crystal structure of Atm1 was the insight into a mutation in ABCB7 that causes the disease, XLSA/A (E433 in human, see below). This residue corresponds to D398 in yeast Atm1, which was identified as one of the key residues required for the interaction with GSH. From the ATPase studies, one might speculate that the GSH-binding site is part of the substrate-binding pocket. Interestingly, key residues identified to interact with GSSG are highly conserved

among atm1, hmt1, Abcb7, and Abcb6 from different species. Other residues found to be mutated in XLSA/A patients were also found in the membrane-spanning domain, E208 in the matrix side (E173 in yeast), whereas I400 and V411 are on the intermembrane side (V365 and V376 in yeast, respectively). The impact of these residues on protein function remains to be tested.

ABCB7 in Human Diseases

ABCB7 loss of function is linked to two human diseases that exhibit ring sideroblasts (erythroblasts with perinuclear iron-loaded mitochondria). To date, four mutations have been identified in the *ABCB7* gene from patients with X-linked sideroblastic anemia with cerebellar ataxia (XLSA/A). However, the reduced levels of *ABCB7* mRNA seen in refractory anemia with ring sideroblasts (RARS) are not due to mutation, suggesting other defects.

X-linked Sideroblastic Anemia with Cerebellar Ataxia (XLSA/A)

XLSA/A is a rare form of congenital sideroblastic anemia, which is characterized by a microcytic anemia with mitochondrial iron accumulation in erythroid precursors of affected males and attributed to defects in ABCB7. While XLSA/A is characterized by neurological defects (e.g., cerebellar ataxia), the anemia and alterations in erythropoiesis resemble those found in patients harboring a defect in the porphyrin biosynthetic enzyme, ALAS2. Despite the neurological defects associated with this disease, *ABCB7* transcripts were not detectable in the adult brain. The four non-synonymous mutations of *ABCB7*, among affected individuals, resulted in amino acid changes of E209D, I400M, V411L, or E433K (Allikmets et al. 1999; Bekri et al. 2000; D'Hooghe et al. 2012; Maguire et al. 2001), which, according to predictions, produce a partial loss of ABCB7 function. Because absence of *Abcb7* in mice is embryonically lethal (suggesting this gene is critical to early development), one infers from this that only mutations with mild effects on ABCB7 function will be tolerated in humans. Consistent with this, yeast harboring the corresponding ABCB7 I400M mutation in Atm1p (analogous to V365M) grew slower (Allikmets et al. 1999). Moreover, yeast expressing mutant Atm1p (D398K) with the corresponding E433K mutation in ABCB7 only produced ~50 % of the normal cytosolic Fe–S cluster proteins (Bekri et al. 2000). The structural studies of Atm1p showed that D398 participates in GSH binding further suggesting the importance of this residue in ABCB7 substrate recognition (Srinivasan et al. 2014). Interestingly, transgenic mice carrying the E433 K allele resulted in male infertility (Pondarre et al. 2007).

Patients affected with XLSA/A exhibit Zn–PPIX accumulation, a rare occurrence, but usually an indication of iron deficiency. The production of Zn–PPIX, instead of heme in iron excess, suggests this iron pool is inaccessible for heme synthesis. In HeLa

cells, knockdown of ABCB7 using siRNA recapitulated some of the phenotypes, including altered iron homeostasis and reduced cytosolic Fe–S cluster proteins (Cavadini et al. 2007). While transferrin receptor expression was upregulated, ferritin, the iron storage molecule, was reduced and ferritin-unbound iron accumulated in mitochondria. The mitochondrial iron was not available for heme formation as evidenced by an increase in PPIX. Unlike yeast ferrochelatase, the mammalian ferrochelatase is an Fe–S cluster-containing protein, and an essential co-factor required for its enzyme activity. ABCB7 and ferrochelatase physically interact in in vitro binding assays, and overexpression of ABCB7 in cells increased both ferrochelatase expression and activity (Taketani et al. 2003). The importance of the interaction between ABCB7 and ferrochelatase requires further investigation because formation of Zn–PPIX in XLSA/A patients implies that ferrochelatase activity is intact, but iron is unexpectedly unavailable when ABCB7 function is impaired.

Refractory Anemia with Ring Sideroblasts

Refractory anemia with ring sideroblasts (RARS) is a type of myelodysplastic syndrome (MDS) characterized by excessive mitochondrial iron accumulation in erythroblasts much like what is seen with XLSA/A patients. Like other forms of MDS, which are heterogeneous but display aberrant hematopoietic cell proliferation and differentiation and loss of circulating blood cells; 10–20 % of RARS progress to acute myeloid leukemia. Although the ring sideroblasts are phenotypically similar to those from XLSA/A patients, no mutations in the *ABCB7* gene, including its promoter, have been identified to date. Nonetheless, *ABCB7* mRNA levels are lower in individuals with RARS compared to healthy controls, or individuals with refractory anemia or refractory anemia with excess blasts (Boultwood et al. 2008). In RARS patients, a splicing factor SFB1 is mutated at a higher frequency (64–90 %), which might explain the reduced *ABCB7* mRNA. Knockdown of ABCB7, using shRNA, in hematopoietic progenitor-enriched bone marrow cells ($CD34^+$), resulted in reduced erythroid progenitors and impaired growth in liquid culture after erythropoietin stimulation, and as expected, impaired erythroid maturation (Nikpour et al. 2013). Conversely, overexpression of ABCB7 gene in $CD34^+$ bone marrow cells from RARS patients enhanced erythroid progenitor formation. While the reduced ABCB7 transcripts appear secondary to SFB1 mutation in RARS, these results further support the role for ABCB7 in erythroid biology.

Abcb7 Knockout Mouse Models Highlight the Importance of This Gene

Mouse models show that constitutive absence of *Abcb7* is embryonically lethal. To overcome this, mice harboring *Abcb7* alleles that can be conditionally deleted were bred to mice harboring *Cre* transgenes that enabled *Abcb7* gene deletion in a

tissue-specific manner (Pondarre et al. 2006). This approach revealed that general deletion of *Abcb7* in embryos prevented them from surviving past day 7.5. Histological analysis of these embryos showed some with growth retardation and hemorrhage; however, there was no evidence for excess iron accumulation. Because of the preferential activation of the female-derived X chromosome in extra-embryonic tissues in mammals, it was speculated that Abcb7 was important for the development of extra-embryonic tissues. This hypothesis was confirmed by crossing *Abcb7* homozygous floxed female mice to males hemizygous for either *Sox-Cre* or *Villin-Cre* transgene, which drives *Cre* expression in embryonic epiblast (sparing much of the extra-embryonic tissues) or in ciliated cells of the extra-embryonic visceral endoderm. Live-born females were obtained from the *Sox-Cre* line but not from the *Villin-Cre* line demonstrating that *Abcb7* is essential in the extra-embryonic tissues during early development.

Abcb7 floxed mice were crossed to inducible- or tissue-specific *Cre* lines to further investigate whether loss of *Abcb7* hemoglobinization during recapitulates human disease. Post-natal deletion of *Abcb7* in the hematopoietic compartment resulted in bone marrow failure and subsequent death within ~20 days (Pondarre et al. 2007). Coupled with this was an early transient siderocytosis, occurring shortly after the deletion of *Abcb7*. These findings support the idea that only mutations resulting in a mild defect in ABCB7 function are tolerated. To determine if an *ABCB7* mutation that was associated with sideroblastic anemia produced a similar phenotype in mice, mice harboring the disease-causing E433 K mutation in *Abcb7* were generated. Notably, red blood cells in these animals were iron-laden (siderocytes), and consistent with anemia as reticulocytes had increased Zn–PPIX compared to Fe–PPIX (heme).

ABCB8

Background

ABCB8 was the first mammalian mitochondrial ABC transporter to be identified and was appropriately named human mitochondrial ABC1 (M-ABC1) (Hogue et al. 1999 JMB). ABCB8, isolated after a cDNA library from a T-cell line, was screened using an EST clone that had nucleotide sequence similarities to the NBD from P-glycoprotein (*ABCB1*). The resulting cDNA encoded a protein with an open reading frame of 718 amino acids that mapped to chromosome 7q35–36. ABCB8 is ubiquitously expressed in human tissues and, like Atm1, contains an N-terminal mitochondrial targeting sequence that mapped to its first 55 amino acids. A cleavable signal sequence accounted for a molecular mass (~60 kDa) that was smaller than predicted. Because inner mitochondrial membrane proteins typically contain cleavable signal sequences, ABCB8 was predicted, like ABCB7 and ABCB10, to reside in the inner mitochondrial membrane. The inner membrane ABC transporters, Atm1p (ABCB7 ortholog) and ABCB10, form homodimers;

therefore, without any obvious potential partners, ABCB8 appears to form a homodimer as a protein complex of an apparent molecular mass of ~120–130 kDa is detected after cross-linking. Unlike ABCB6 and Atm1p, the conserved Walker A motif is critical for the stability of ABCB8, as point mutations in key residues in this motif rendered ABCB8 undetectable after transient expression in COS-7 cells.

ABCB8 as a Part of MitoK_{ATP} Channel

The physiological substrate of ABCB8 has not been determined, yet. However, ABCB8 protects against oxidative stress. This unanticipated finding was revealed after the discovery of ABCB8 in a macromolecular complex encompassed in the mitochondrial ATP-sensitive K^+ channel (mitoK_{ATP}) (Ardehali et al. 2004). Activation of mitoK_{ATP} protected cardiac and neuronal cells against oxidant-induced apoptosis during brief ischemic episodes. However, ABCB8 role is unclear as the mitochondrial enzyme, succinate dehydrogenase (SDH), is also a component of this complex. Further, immunoprecipitation of SDH from inner mitochondrial membranes revealed additional interaction between SDH, ABCB8, and three more mitochondrial proteins, phosphate carrier (PIC), adenine nucleotide translocator (ANT), and ATP synthase (ATPase). PIC and ANT have been suggested to exhibit ion channel activity, thus it is conceivable that ABCB8 regulates the mitoKATP. This would be analogous to the sulfonylurea receptors' (SUR, ABCC8/9) regulation of the plasma membrane K_{ATP} channel. At this point, how ABCB8 regulates mitoK_{ATP} is unclear, but warrants further investigation.

ABCB8 and Iron Modulation

As discussed above, mitoK_{ATP} activity protects against oxidative stress (e.g., ischemia). ABCB8 role under such stress was directly tested using a ROS-generating system. In this system, knockdown of ABCB8 in ROS-exposed neonatal rat cardiomyocytes resulted in a loss of mitochondrial membrane potential and cell viability (Ardehali et al. 2005). In contrast, ABCB8 overexpression protected the cells against ROS-mediated mitochondrial injury. This protection appears unrelated to the mitoK_{ATP} channel as its inhibition prior to ROS formation did not alter the mitochondrial membrane potential. Therefore, ABCB8 protects against ROS independent of mitoK_{ATP}. Modulation of mitochondrial iron levels might be how ABCB8 protects against oxidative stress (see below).

The function of ABCB8 in cardiomyocytes was further investigated by generating a cardiac-specific *Abcb8*-null mouse model (Ichikawa et al. 2012). Hearts deficient in *Abcb8* exhibited multiple defects, including reduced heart contractility and cardiac output, the latter being consistent with defects in systolic and diastolic function. While no gross alteration in heart morphology was observed, electron

microscopic examination of *Abcb8*-null hearts revealed aberrant mitochondrial structures. The mitochondria were smaller and cristae morphology was abnormal. Furthermore, mitochondria contained electron-dense material similar to those of animals exposed to iron loading. Indeed, mitochondrial iron levels modestly increased in *Abcb8*-null hearts. These in vivo findings were supported by knockdown of *Abcb8* in cultured cardiomyocytes. Abcb8 deficient cardiomyocytes had enhanced mitochondrial iron accumulation, whereas Abcb8 overexpression reduced mitochondrial iron levels. Subsequent studies, using radiolabeled iron, suggested iron export was impaired after knockdown of ABCB8, although further experiments are needed to determine what form of iron is exported (i.e., is it heme or an Fe–S cluster?). Notably, mitochondrial membrane potential was unaffected in these Abcb8 siRNA-treated cells, suggesting that the mitochondrial injury seen in the *Abcb8*-null mouse heart is likely due to chronic iron accumulation in the mitochondria. Surprisingly, the activity of cytosolic Fe–S cluster proteins, such as xanthine oxidase and aconitase, was reduced in either the *Abcb8*-null mouse heart or siRNA-treated cardiomyocytes. So it is conceivable that ABCB8 not only regulates iron export, but Fe–S cluster assembly, too. Whether ABCB8 is directly involved in iron transport or regulates another transporter of mitochondrial iron remains to be seen. It is of interest that both *Abcb7* and *Abcb8* knockout mice have some overlapping characteristics (i.e., mitochondrial iron accumulation and reduced cytosolic Fe–S cluster proteins), suggesting they share redundant function in modulating mitochondrial iron levels. However, how ABCB8 regulates iron homeostasis remains a mystery. Moreover, without direct determination of heme (there was no change in PPIX levels), it is difficult to gain insight into how *Abcb8* deficiency affects mitochondrial iron homeostasis.

ABCB8 Protects Against Doxorubicin-Induced Oxidative Stress

Doxorubicin is a widely used cancer chemotherapeutic with cardiomyopathy as an unfortunate side effect. This is independent of its primary mechanism of inhibiting topoisomerase. One hypothesized mechanism of doxorubicin-induced cardiomyopathy is the “ROS and iron” hypothesis. This was supported by the finding that the iron chelator dexrazoxane (DXZ) protected against doxorubicin-induced cardiomyopathy. In further support, an animal model shows doxorubicin-mediated heart injury is related to greater systemic iron absorption. Notably, ABCB8 knockdown sensitized cardiomyocytes to doxorubicin toxicity, accompanied by an increased cellular ROS. It is possible, by regulating mitochondrial iron, ABCB8 may indirectly protect against doxorubicin toxicity, an idea supported by knockdown of ABCB8, which enhances doxorubicin cytotoxicity in normally resistant melanoma cells.

ABCB10

Background

ABCB10, first identified as a target of GATA-1, (a transcription factor essential for differentiation of erythroid cells), was initially named ABC-me for mitochondrial erythroid transporter (Shirihai et al. 2000). The gene (*ABCB10, ABC-me, M-ABC2, MTABC2*) was localized to chromosome 1q32 in man and encodes a protein of 716 amino acids. ABCB10 is highly expressed at sites of definitive hematopoiesis such as fetal liver erythroid cells (i.e., $Ter119^{+}$) and adult bone marrow (Fig. 2). In adult mice, *Abcb10* is also highly expressed in other tissues including liver, kidney, and heart, and, to a lower extent, in brain and spleen.

Biochemical Characteristics of ABCB10

The biochemical properties of ABCB10 have been extensively studied, including a well-characterized mitochondrial targeting signal that is crucial to ABCB10 localization to the mitochondrial inner membrane with its C-terminal NBD facing the matrix (Graf et al. 2004). The mitochondrial targeting sequence comprises the first 105 amino acids, which, upon cleavage, produce a protein with an apparent molecular mass of 65 kDa instead of a predicted 78 kDa. The deletion of this N-terminal signal sequence abolished its mitochondrial localization.

Mass spectrometry revealed that ABCB10 is a homodimer (Graf et al. 2004), and the crystal structure of ABCB10 in a nucleotide-free state showed an open inward-facing conformation in agreement with previously studied ABC transporters (Shintre et al. 2013). The ABCB10 protein fold is similar to those of other ABC transporters, where α-helices from each monomer are intertwined with each other. Unexpectedly, ABCB10 maintained an open inward-facing conformation when a non-hydrolyzable ATP analog is bound. This finding contrasted with the classic model of substrate transport whereby the binding of nucleotide induced a dynamic conformational change to yield an outward-facing conformation.

Role of ABCB10 in Erythroid Biology

ABCB10 has a crucial role in erythroid development as constitutive deletion produced anemic embryos that failed to thrive (embryos died by gestational day 12.5 accompanied by apoptotic erythroid precursors) (Hyde et al. 2012). To determine whether Abcb10 was required for erythrogenisis, red blood cell differentiation markers CD71 (aka transferrin receptor) and TER119 were analyzed in wild-type and *Abcb10*-null embryos. During erythrogenesis, CD71 expression increased,

followed by TER119 expression. Because CD71 is involved in iron acquisition, it is noteworthy that *Abcb10*-null embryos have dramatically reduced CD71 positive cells. Iron is crucial for hemoglobin biogenesis; therefore, it is not surprising that *Abcb10*-null erythroid differentiation is stalled with increased apoptosis at the peak of hemoglobin synthesis. ABCB10 role in hemoglobin synthesis appears conserved in humans and mice as knockdown of ABCB10 reduced hemoglobinization during differentiation in the human erythroleukemia cell line K562 (Tang et al. 2012). ABCB10 role in erythrogenesis is not only confined to the embryonic stage; deletion of *Abcb10* from the hematopoietic cells of adult mice harboring floxed *Abcb10* mice resulted in a progressive anemia characterized both by reduced red blood numbers and red cells exhibiting impaired hemoglobin and heme production (Yamamoto et al. 2014). The defect in heme biosynthesis was accompanied by an elevation in reticulocyte PPIX. These results indicate ABCB10 regulation of heme biosynthesis is crucial to erythroid differentiation.

Although ABCB10 was initially proposed as a mitochondria exporter of heme (Shirihai et al. 2000), more recent studies suggest that ABCB10 might regulate the terminal steps in heme biosynthesis through an interaction with other mitochondrial proteins (Chen et al. 2009, 2010). In differentiated mouse erythroleukemia (MEL) cells, ABCB10 co-immunoprecipitated with mitoferrin (Slc25a37), a mitochondrial iron importer (Chen et al. 2009). The stability and activity of mitoferrin are increased by the interaction with ABCB10. Subsequent studies using mass spectrometry identified ferrochelatase (the terminal enzyme in heme biosynthesis) as part of an oligomeric protein complex containing ABCB10, mitoferrin, and ferrochelatase (Chen et al. 2010).

Based on ABCB10 knockdown in cardiac myoblasts exhibiting reduced porphyrins including heme and hemoproteins (Liesa et al. 2011), an alternative role for ABCB10 was proposed. The authors suggested that ABCB10 increased the export of ALA out of the mitochondria because ALA augmentation normalized porphyrin levels in ABCB10 knockdown cells. A caveat to this interpretation is that these studies did not directly measure transport of ALA by ABCB10. Therefore, how ABCB10 modulates heme biosynthesis is not clear: is it at the terminal step or the beginning?

ABCB10 Role in Protection Against Oxidative Stress

ABCB10 expression protected against oxidative stress in yeast and mice, which is not surprising given aberrant accumulation of heme precursors is related to oxidative stress. In yeast, Mdl1p, an ABCB10 ortholog (Mdl1 shares sequence homology to ABCB8 to a lesser extent), conferred resistance to oxidative stress and heavy metal toxicity (Chloupkova et al. 2003). Accordingly, *Abcb10*-null embryos exhibit mitochondrial ROS and protein carbonylation in red blood cells (Hyde et al. 2012), which appears to be related to reduced erythroid differentiation capacity. Notably, quenching ROS with a superoxide dismutase 2 mimetic rescued erythroid

differentiation. In humans, differentiation of erythroid colonies was also impaired by *ABCB10* knockdown (Yamamoto et al. 2014), suggesting a conserved function among different species.

ABCB10 role in protection against oxidative stress extends beyond the erythroid compartment. For example, loss of one allele of *Abcb10* in mice resulted in reduced recovery of cardiac function after ischemic perfusion (Liesa et al. 2011). Mitochondrial defects consistent with increased oxidative stress accompanied the defect. Furthermore, attenuation of oxidative damage by the superoxide dismutase/catalase mimetic, EUK-203, restored cardiac function to wild-type levels after the ischemic insult.

Conclusion and Future Perspective

In the last decade, animal models lacking each of the four mitochondrial ABC transporters have been established, all of which have highlighted the importance of these proteins in the health and/or function of the mitochondria. Although the precise roles for some of them remain elusive, largely because substrate identification has lagged, the physiological roles of these transporters are emerging. Notably, there are several common threads that tie these transporters together: (a) role in erythroid biology through iron and heme homeostasis and (b) protection against oxidative stress.

ABCB6, ABCB7, and ABCB10 are upregulated during erythroid differentiation with each having some similar, but non-overlapping roles in erythroid biology. ABCB7 and ABCB10 are essential for normal erythropoiesis, whereas ABCB6 role was only evident under stress erythropoiesis. ABCB6 and ABCB10 are both involved in heme and hemoprotein homeostasis, where ABCB6 shuttles porphyrins into mitochondria. ABCB10 role in heme biosynthesis is unclear, with either a role in the terminal step in heme biosynthesis or export of ALA into the cytoplasm. Although exact substrates are yet to be identified, ABCB7 role in maintaining iron homeostasis through Fe–S cluster biogenesis is evident. Notably, loss of ABCB6, ABCB7, ABCB8, and ABCB10 all results in increased sensitivity to oxidative stress. Because their homologs, especially Atm1p and Hmt1p, have been shown to alleviate heavy metal toxicity and oxidative stress in different organisms, it is plausible that their mammalian counterparts also have a role in heavy metal detoxification. Currently, ABCB6 and ABCB8 have been implicated in modulating drug toxicity for artemisinin and doxorubicin, respectively. Future studies will undoubtedly address how these transporters affect and modify disease states.

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Human ABC Transporter ABCC11: Looking Back Pioneers' Odyssey and Creating a New Path Toward Clinical Application

Toshihisa Ishikawa and Yu Toyoda

Abstract In 1969 pioneering work was made by Srivastava and Beutler (J Biol Chem 244:9–16, 1969) who reported that elimination of GSSG from human erythrocytes to the incubation medium is a unidirectional and energy-dependent process. Since then, a large number of biochemical/physiological studies were performed to characterize that energy-dependent unidirectional transporter. In 1992 Cole et al. made a giant leap in identifying molecular nature of that transporter to name MRP1 (ABCC1 according to the international nomenclature). Thereafter, completion of the Human Genome Project and advanced bioinformatics technology enabled researchers to gain more insight into the gene structure of a total of 48 ABC transporters in the human genome. Today, rapid growth of personalized medicine is being supported by emerging new technologies together with accumulating knowledge of pharmacogenomics. In this chapter, we show technological developments hitherto we made and will introduce a new SNP-typing method for human *ABCC11* gene that is expected to provide a practical tool for clinical diagnosis of axillary osmidrosis.

Keywords ABC transporter · ABCC11 · Glutathione · GS-X pump · MRP · Oxidative stress · Personalized medicine · Apocrine gland · Axillary osmidrosis · Earwax

Introduction

In aerobic living cells on the earth, there is a balance between oxidants and antioxidants, or reduction–oxidation (redox) homeostasis under normal physiological conditions. Reactive oxygen species, such as superoxide anion radical ($^{\cdot}O_2^-$),

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hydroxyl radical ($^{\cdot}OH$), and hydrogen peroxide (H_2O_2), are continuously generated within living cells and play important roles in many cellular pathophysiological functions, including inflammation, apoptosis, mutation, and aging (Finkel 2003). Cytotoxic agents, including antitumor agents, xenobiotics, and carcinogens, induce oxidative stress. One of the important strategies in counteracting these stresses is the glutathione (GSH) system (Winyard et al. 2005). Under oxidative stress, GSH is oxidized by GSH peroxidase to glutathione disulfide (GSSG), which is eliminated by members of the GS-X pump family, commonly known as multidrug resistance protein (MRP) efflux proteins. Excess GSSG can be catalytically reduced back to GSH by NADPH-dependent GSSG reductase (Fig. 1). GSH also regulates the activities and biosynthesis of other redox-regulating enzymes such as superoxide dismutase (SOD) and DT-diaphorases (NQO1 and NQO2). Because of its abundance (1–10 mM inside cells) (Hwang et al. 1992; Smith et al. 1996), the GSH/GSSG system represents a major pathway for redox regulation in the cells.

GSH is a ubiquitous tripeptide thiol (L-γ-glutamyl-L-cysteinyl-glycine) that is present in virtually all eukaryotes (Meister and Anderson 1983). It is a vital intra- and extracellular cytoprotectant (Droge 2002; Reed 1990) and an effective scavenger of reactive oxygen species (ROS). It is estimated that GSH biosynthesis originated about 3.5 billion years ago. GSH is found in the vast majority of

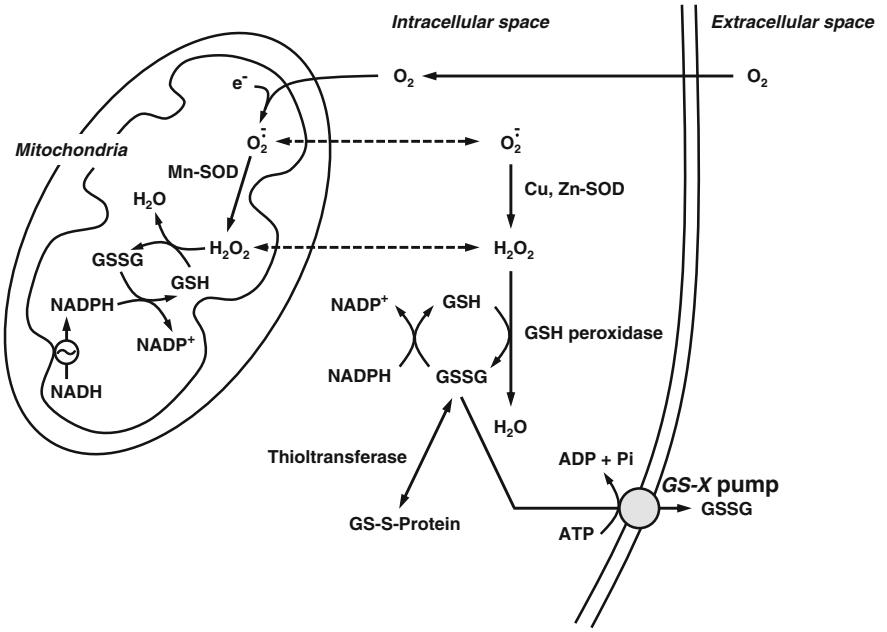

Fig. 1 Role of the GS-X pump transporter in the regulation of intracellular redox conditions. Under oxidative conditions, GSH is oxidized to GSSG by GSH peroxidase. GSSG can interact with cellular proteins to form glutathione-mixed sulfide (GS-S-protein). GSSG is either eliminated by the GS-X pump in an ATP-dependent manner or reduced back to GSH by GSSG reductase

eukaryotes, whereas in eubacteria, GSH biosynthesis is limited to only two groups, i.e., cyanobacteria and purple bacteria (Fahey and Sundquist 1991). The former appeared on earth about 3.5 billion years ago and was capable of oxygenic photosynthesis. The cyanobacteria are considered to have given rise to plant chloroplasts, whereas the purple bacteria are considered to have introduced the ancestor responsible for eukaryotic mitochondria. GSH production appears to be closely associated with those prokaryotes responsible for the oxygen-producing and oxygen-utilizing pathways of eukaryotes, suggesting that the ability of GSH biosynthesis may have been acquired by eukaryotes during the endosymbiotic process that give rise to chloroplasts and mitochondria (Fahey and Sundquist 1991). In fact, GSH plays a pivotal role in the protection of living cells under conditions of oxidative stress (Chance et al. 1979; Sies 1985).

GSH also serves as a cofactor for conjugation reactions involved in the elimination of various xenobiotics such as chemical carcinogens, environmental pollutants, and antitumor agents (Sies 1988). The formation of hydrophilic glutathionyl conjugates is catalyzed by glutathione S-transferases (GSTs), a family of so-called phase II enzymes that mediate the conjugation reaction in a substrate-dependent fashion (Ishikawa 1992; Hayes et al. 2005; Townsend and Tew 2003). These GST isoenzymes are also involved in the endogenous biosynthesis of leukotrienes, prostaglandins, testosterone, and progesterone. Among their substrates, GSTs conjugate 4-hydroxynonenal, leukotriene A_4, and the signaling molecules 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15Δ-PGJ_2) and Δ^7-prostaglandin A_1 (Ishikawa 1992; Ishikawa et al. 1998; Suzuki et al. 1997).

Plant and animal cells eliminate a broad range of lipophilic toxins from the cytosol following their conjugation with glutathione (GSH) (Ishikawa 1992; Ishikawa et al. 1997; Li et al. 1995; Martinoia et al. 1993; Rea et al. 2003). This transport process is mediated by GS-X pumps, namely organic anion transporters with Mg^{2+}-ATPase activity. The term "GS-X pump" was originally proposed based on its transport activity and high affinity for glutathione S-conjugates (GS-conjugates), glutathione disulfide (GSSG), and cysteinyl leukotrienes (Ishikawa 1992). This pump was thought to shuffle these glutathionyl metabolites into the appropriate cellular compartments (Ishikawa 1992; Ishikawa et al. 1997). Accumulated data has suggested that the GS-X pump exhibits broad substrate specificity toward different types of organic anions and thereby play a physiologically important role in inflammation, oxidative stress, xenobiotic metabolism, and antitumor drug resistance.

Pioneers' Odyssey to Discover GS-X Pump

In 1969, pioneering work was carried out by Srivastava and Beutler (1969) who reported that elimination of GSSG from human erythrocytes to the incubation medium is a unidirectional and energy-dependent process (Srivastava and Beutler 1969) (see Table 1 for historical background). GSSG transport occurred even against a concentration gradient of GSSG, and the transport was halted almost

Table 1 Identification of C-class ABC transporter genes

1969	The first report on energy-dependent GSSG elimination from human erythrocytes	Srivastava and Beutler
1980	ATP-dependent GSSG transport in inside-out membrane vesicles of erythrocytes	Kondo et al.
1981	ATP-dependent GSSG transport in inside-out membrane vesicles of erythrocytes	Board
1982	ATP-dependent transport of GS-conjugate in erythrocyte membranes	Kondo et al.
1982	GSSG and GS-conjugate release from isolated perfused rat liver to bile	Akerboom et al.
1984	GSSG and GS-conjugate release from isolated perfused rat heart	Ishikawa and Sies
1986	ATP-dependent transport of GS-conjugate in erythrocyte membranes	Labelle et al.
1986a, b	Energy-dependent GSSG transport in rat heart measured by in vivo NMR	Ishikawa et al.
1987,	Discovery of transport-deficient rat that lacks transport of bilirubin-glucuronide conjugates	Jansen et al.
1987	Deficient hepatobiliary transport of cysteinyl leukotrienes in TR^- rats	Huber et al.
1989	Deficient hepatobiliary transport of GSH, GSSG, and GS-conjugates in TR^- rats	Elferink et al.
1989	ATP-dependent transport of GS-conjugate in plasma membrane vesicles from rat heart	Ishikawa
1990	ATP-dependent transport of GS-conjugate in plasma membrane vesicles from rat liver	Kobayashi et al.
1990	ATP-dependent transport of leukotriene C_4 in rat liver and heart membrane vesicles	Ishikawa et al.
1991	Discovery of transport-deficient EHBR lacking hepatobiliary transport of GS-conjugate	Takikawa et al.
1992	Definition of GS-X pump and phase III system for xenobiotic metabolism	Ishikawa
1992	Cloning of MRP1 cDNA from doxorubicin-resistant human lung cancer cell line	Cole et al.
1994	Discovery of leukotriene C_4 and GS-DNP transport by MRP1	Leier et al.
		Muller et al.
1996	Cloning of Mrp2/cMOAT cDNA from the liver cDNA library of TR^- rats	Paulusma et al.
1997	Identification of MRP3, 4, and 5 in human cancer cell lines	Kool et al.
1999	Identification of MRP6 in human tissues and cancer cell lines	Kool et al.
2001	Identification of MRP7 in human tissues	Hopper et al.
2001	Identification of MRP8 and MRP9 in human adult liver	Yabuuchi et al.
		Bera et al.
		Tammur et al.
2002	Finding of ABCC13 as pseudogene	Yabuuchi et al.

entirely when endogenous ATP was depleted by preliminary incubation of erythrocytes in a glucose-free medium for 8 h or by the presence of fluoride in the incubation medium. Their report provided the first evidence that GSSG efflux is mediated not by simple diffusion but rather by active membrane transport. Although they suggested the energy dependence of GSSG transport, the direct requirement of ATP was not elucidated. In 1980, the transport of GSSG across the plasma membrane was proven to be an ATP-dependent "primary" active process in inside-out membrane vesicles from human erythrocytes (Kondo et al. 1980). Subsequently, ATP-dependent GSH conjugate transport was demonstrated by Board (1981), Kondo et al. (1982), and Labelle et al. (1986).

In 1984, Ishikawa and Sies reported that GSSG and GS-conjugates were released from the isolated perfused heart (Ishikawa and Sies 1984) (Fig. 2). Because the heart is continuously exposed to highly oxygenated blood from the lungs, cardiomyopathy may result from oxidative damage inflicted by hyperoxia or administration of certain drugs. Thus, GSSG efflux was thought to be an important defense mechanism against oxidative stress (Fig. 1). To characterize the GSSG efflux mechanism, we constructed a rat heart perfusion system, where electrocardiogram (ECG), heart beat rate, left ventricular presser (LVP), and oxygen consumption rate were observed to ensure that the cardiac function was normal (Fig. 2). When we correlated the GSSG efflux rate with the cellular GSSG level in rat hearts exposed to oxidative stress (*t*-butyl hydroperoxide), saturation kinetics was observed between these two parameters with an apparent Km value of 30 nmol × g of heart^{-1} and the maximal GSSG efflux rate 7.5 nmol × min^{-1} × g of heart^{-1}. This

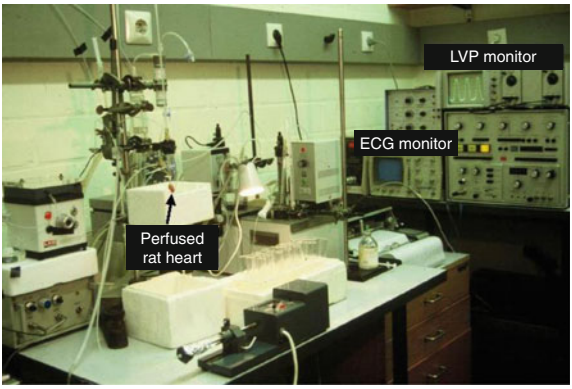

Fig. 2 Detection of GSSG efflux from isolated perfused rat hearts. Hearts from male Wistar rats of 180–250 g body weight were perfused by the method of Langendorff at 37 °C with a constant load of 80 cm of water pressure (Ishikawa and Sies 1984; Ishikawa et al. 1986a, b). Standard perfusion medium was Krebs–Henseleit bicarbonate buffer solution containing 2.5 mM $CaCl_2$ and 5.5 mM glucose equilibrated with 95 % O_2 and 5 % CO_2. Effluent perfusate was collected every minute into plastic tubes maintained at 0 °C for the measurement of GSSG. GSH and GSH levels in the effluent perfusate as well as in the heart tissue were biochemically measured as described previously (Ishikawa and Sies 1984)

finding strongly inspired us to imagine that the GSSG efflux in the heart is mediated by a certain membrane carrier (Ishikawa and Sies 1984).

The heart can undergo serious pathological states associated with peroxidation of polyunsaturated fatty acids in biomembranes. Hydroxyalkenals and other diffusible reactive short-chain carbonyl compounds are considered of major importance in the cause of cytotoxicity. The hydroxyalkenals with α,β-unsaturated carbonyl structure are electrophilic and react rapidly with thiol groups of GSH, cysteine, coenzyme A, and proteins. Ishikawa et al. (1986a) reported that the glutathione S-conjugate of 4-hydroxynonenal is effectively eliminated from the heart by the action of the GS-X pump (Ishikawa et al. 1986a). Based on kinetic analysis in isolated perfused rat heart, the existence of a common export pump for both GSSG and glutathione S-conjugates is suggested.

To gain more insight into the ATP-dependent GSSG efflux from the heart, we performed in vivo nuclear magnetic resonance (NMR) measurement with an isolated perfused rat heart. Figure 3 demonstrates an in vivo NMR system where cytosolic free ATP/ADP ratio and cellular pH level were continuously monitored. The relationship of GSSG efflux rate versus cytosolic free ATP/ADP ratio shows that GSSG efflux rate is half-maximal at (ATP/ADP) free = 10 (Ishikawa et al. 1986b), suggesting that GSSG efflux is an ATP-dependent transport process. GSSG efflux from the heart is not affected by epinephrine, norepinephrine, or dibutyryl cyclic AMP; GSSG transport is independent of α- or β-adrenergic hormonal regulations (Ishikawa et al. 1986b).

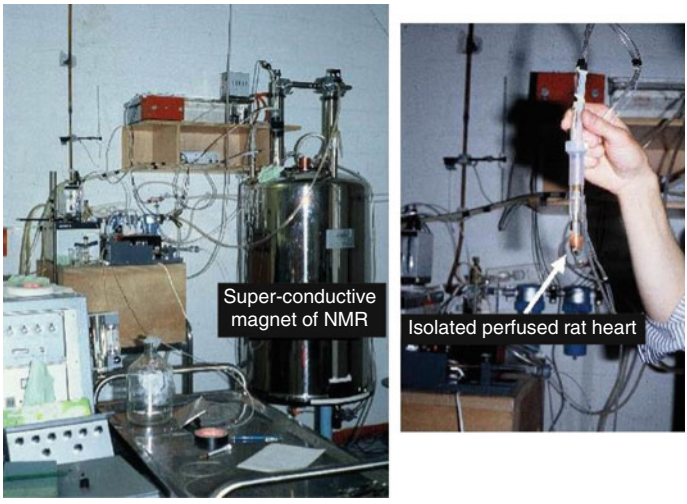

Fig. 3 Direct measurement of ATP-dependent GSSG efflux by ^{31}P-NMR spectroscopy. ^{31}P-NMR spectra of isolated perfused hearts were recorded with a Bruker WH 360 NMR spectrometer operated in the Fourier transform mode. The heart was placed in a 15-mm NMR sample tube and inserted into a probe which was seated in the bore of a superconducting magnet (8.5 T). NMR spectra were taken by 80 scans of a 60° broadband pulse with a 0.6 s interpulse delay. The intracellular pH was determined from the chemical shift of inorganic phosphate signal as described previously (Ishikawa et al. 1986b)

Furthermore, using plasma membrane vesicles prepared from rat hearts, Ishikawa (1989) provided direct evidence for ATP-dependent primary active transport of GSSG and GS-conjugates. The cardiac GS-X pump was shown to have high affinity for GS-conjugates with long aliphatic carbon chains (Ishikawa et al. 1986b). Leukotriene C4 (LTC4), a pro-inflammatory mediator, was found to be an endogenous substrate for the GS-X pump (Ishikawa et al. 1989, 1990).

Technological Development to Advance Transport Studies

It is important to note that Ishikawa (1989) developed the standard method to characterize ATP-dependent transport mediated by GS-X pumps, namely C-class ABC transporters. Plasma membrane fractions from many different types of cells exhibit extremely high activities of ecto-ATPases that catabolize nucleotide triphosphates (e.g., ATP, GTP, and CTP). Because of this activity, ATP in the reaction medium is rapidly degraded during transport experiments. Thus, the ATP concentration in the reaction medium must be maintained constant for a sufficient period of the transport experiment. To solve this problem, Ishikawa (1989) introduced an ATP-regenerating system by adding creatine kinase and creatine phosphate into the transport reaction medium. Accordingly, in the case of plasma membrane vesicles from rat hearts, ATP concentration was maintained almost constant for at least 20 min of the transport experiment (Ishikawa 1989).

At that time, however, the throughput of transport experiments was quite low, because whole experiments absolutely depended on manual procedures. It was urgently needed to enhance the throughput of transport experiments by creating a new automated system. Ishikawa et al. (2005) developed a semi-automated system for transport experiments. The high-speed screening system using 96-well plates (Fig. 4) enabled to analyze the substrate specificity of ABC transporters and their genetic variants (Ishikawa et al. 2005). As we will discuss later, the effect of genetic polymorphisms on the transport activity depends on the substrates tested, and therefore the functional analysis using a wide variety of substrates is of great interest. One amino acid substitution can alter interactions between the active site of an ABC transporter and substrate molecules. Therefore, it was important to quantitatively analyze and to evaluate such structure-related interactions. In this context, the high-speed screening system provided a powerful tool to quantify the impact of genetic polymorphisms on the function of ABC transporters (Ishikawa et al. 2005).

Furthermore, biological materials for transport experiments emerged as another critical issue, since the throughput of transport experiments was significantly enhanced by introducing the high-speed screening method. Preparation of plasma membrane vesicles from human tissues at a large amount was not realistic at all. A new approach was needed. Today, isolated membrane vesicles from insect cells provide a practical tool for low cost and high-throughput analysis of ABC transporters (Saito et al. 2009). Baculovirus-infected insect cells have been successfully

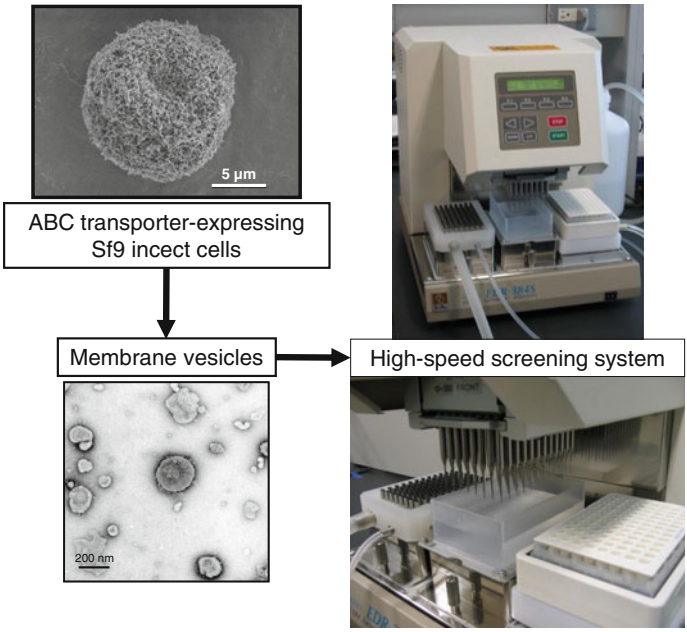

Fig. 4 High-speed screening for assessment of the function of ABC transporter expressed in Sf9 insect cell membranes. ABC transporter of interest was expressed in Sf9 insect cells by using recombinant virus, and plasma membrane vesicles were prepared from Sf9 cells, as described previously (Ishikawa et al. 2005). Membrane vesicles are formed by passing the membrane suspension through a 27-gauge needle. To measure the ATP-dependent transport, the standard incubation medium should contain plasma membrane vesicles (10 or 50 μg of protein), 1–200 μM radio-labeled substrate (e.g., [^{3}H]methotrexate or [^{3}H]dinitrophenyl glutathione), 0.25 M sucrose, 10 mM Tris/Hepes, pH 7.4, 10 mM MgCl2, 1 mM ATP, 10 mM creatine phosphate, and 100 μg/ml of creatine kinase in a final volume of 100 μl. The incubation was carried out at 37 °C. After a specified time (20 min for the standard condition), the reaction medium was mixed with 1 ml of the ice-cold stop solution (0.25 M sucrose, 10 mM Tris/Hepes, pH 7.4, and 2 mM EDTA) to terminate the transport reaction. Subsequently, aliquots (280 μl per well) of the resulting mixture were transferred to MultiScreen^{TM} plates (Millipore). Under aspiration, each well of the plate was rinsed with the 0.25 M sucrose solution containing 10 mM Tris/Hepes, pH 7.4, four times (4 × 200 μl for each well) in an EDR384S system (BioTec, Tokyo, Japan). The radio-labeled substrate thus incorporated into the vesicles was measured by counting the radioactivity remaining on the filter of MultiScreen^{TM} plates, where each filter was placed in 2 ml of liquid scintillation fluid (Ultima Gold, Packard BioScience). Detailed procedures are described in Ishikawa et al. (2005)

employed to give relatively high protein expression yields; for example, *Spodoptera frugiperda* (Sf9) cells are widely used to obtain membranes overexpressing various ABC transporters. We usually infect Sf9 cells (1×10^6 cells/ml) with human ABC transporter-recombinant baculovirus and culture them at 26 °C with gentle shaking. The baculovirus has a 130-kb double-stranded DNA genome, packaged in a cigar-shaped (25 by 260 nm) enveloped nucleocapsid. Baculovirus enters insect cells via receptor-mediated endocytosis. The viral fusion protein gp64

is responsible for acid-induced endosomal escape. In the cytoplasm, the nucleocapsid probably induces the formation of actin filaments, which provide a possible mode of transport toward the nucleus.

The use of low ionic strength buffers during the membrane preparation steps promotes the formation of open membrane sheets and inside-out membrane vesicles. It is important to maintain high integrity of the plasma membrane vesicles used in the transport assay. In other words, the membrane vesicles have to be completely sealed. To examine the quality of plasma membrane vesicles prepared from Sf9 cells, Saito et al. (2009) used electron microscopy (TEM and SEM; Fig. 4) technologies and identified the optimal conditions required to prepare the membrane vesicles. The timing of harvesting Sf9 cells after baculovirus infection appears to be very critical. The membrane morphology of infected Sf9 cells changed greatly; in particular, numerous pores were observed in the cell membrane on day 5. Membrane vesicles prepared from those cells (day 5) are useless for transport experiments. It is also important to prepare membrane vesicles in the presence of serine/cysteine protease inhibitors. Leupeptin (10 μg/ml) inhibited the degradation of ABC transporter protein in membrane vesicles prepared from baculovirus-infected Sf9 cells during repetitive freeze–thaw cycles.

Membrane vesicles (suspended in 250 mM sucrose and 10 mM Tris/Hepes, pH 7.4) can be stored at −80 °C or in liquid nitrogen until used. For long-term (over one year) storage of membrane vesicles, however, it is recommended to substitute tolehalose for sucrose in the membrane vesicle preparations. Trehalose (α-D-glucopyranosyl α-D-glucopyranoside) is a non-reducing disaccharide composed of two glucose molecules joined by an α,α-1,1 linkage. Trehalose is a stress protectant in biological systems as it interacts with and directly protects lipid membranes and proteins from desiccation and during freezing (Saito et al. 2009).

Discovery of *ABCC1 (MRP1)* Gene Encoding GS-X Pump

In 1992, Cole et al. made a giant leap in identification of the molecular nature of GS-X pump (Table 1). They used a differential screening approach to clone a cDNA encoding a deduced protein of 190 kDa, which they named "MRP1". This protein was overexpressed in a doxorubicin-resistant cell line (Cole et al. 1992). Sequence analyses of this MRP1 cDNA revealed it to be an ABC transporter, with closer sequence similarity to cystic fibrosis transmembrane conductance regulator (CFTR/ABCC7) than to P-glycoprotein-encoded MDR1 (ABCB1). Further studies demonstrated that membrane vesicles prepared from MRP1 (ABCC1)-transfected cells exhibited elevated transport activities for GSH-conjugates, such as LTC_4 and S-(2,4-dinitrophenyl)-glutathione (GS-DNP) (Leier et al. 1994; Muller et al. 1994), the steroid conjugate 17-β-glucuronosyl [^{3}H]estradiol, and bile salt conjugates 6-α-[^{14}C]glucuronosylhypodeoxycholate and 3-α-sulfatolithocholyl [^{3}H]taurine (Deeley and Cole 2006; Jedlitschky et al. 1996). Finally, these studies provided direct evidence that MRP1 (ABCC1) was the long-sought GS-X pump.

Thereafter, completion of the Human Genome project and advances in bioinformatics enabled researchers to identify a total of 48 ABC transporters in the human genome. It has been reported that mutations of ABC protein genes are causative in several genetic disorders in humans (Dean et al. 2001). Many of human ABC proteins are involved in membrane transport of drugs, xenobiotics, endogenous substances, or ions, thereby exhibiting a wide spectrum of biological functions. Based on the arrangement of molecular structural components, i.e., nucleotide-binding domains and topologies of transmembrane domains, the hitherto reported human ABC proteins are classified into seven subfamilies (A to G). The HUGO Human Gene Nomenclature Committee developed a standard nomenclature for the human ABC-transporter gene family.

It is now known that MRPs belong to the C-class of the human ABC protein gene family, according to the HUGO Human Gene Nomenclature. This C-class contains a total of 12 genes, of which nine are MRP-related and designed MRP1 through MRP9 (ABCC1-6 and ABCC10-12, respectively) (Fig. 5a). The other three are the cystic fibrosis transmembrane conductance regulator (CFTR/ABCC7) and the sulfonylurea receptors (SUR1/ABCC8 and SUR2/ABCC9). All of the members belonging to the C-class contain two ATP-binding cassettes in the intracellular part and two core membrane-spanning domains MSD_1 and MSD_2. In addition, ABCC1-3, 6, and 10 contain one additional membrane-spanning domain (MSD_0) (Fig. 5b). Topologically, MSD_0 is not essential for the catalytic function of ABCC1 (MRP1), because deleting this domain does not compromise its LTC_4 transport activity (Bakos et al. 1998). Mutations at certain Cys residues within MSD_0, however, substantially reduce LTC_4 transport activities (Leslie et al. 2003; Yang et al. 2002), and a photoaffinity labeling study demonstrated that MSD_0 interacts with a photoreactive azido analog of LTC_4 (Karwatsky et al.2005) suggesting that MSD_0 may not be entirely functionless. The C-class ABC transporters, in particular MRPs, were hitherto reviewed in many excellent articles (Borst and Elferink 2002; Borst et al. 2000, 2006; Deeley and Cole 2006; Kruh and Belinsky 2003; Kruh et al. 2007). In this article, therefore, we will provide clinical aspects focusing on ABCC11 (MRP8).

Genetic Polymorphisms in *ABCC11 (MRP8)* Gene

The *ABCC11* (*MRP8*) gene was identified by searching the human genome database. In 2001, three research groups independently cloned ABCC11 (MRP8) together with ABCC12 (MRP9) from the cDNA library of human adult liver (Yabuuchi et al. 2001; Bera et al. 2001; Tammur et al. 2001). Both *ABCC11* (*MRP8*) and *ABCC12* (*MRP9*) genes are located on human chromosome 16q12.1 in a tail-to-head orientation with a separation distance of about 20 kb (Fig. 6). The predicted amino acid sequences of both gene products show a high similarity to those of ABCC4 (MRP4) and ABCC5 (MRP5) (Fig. 6). However, there is no

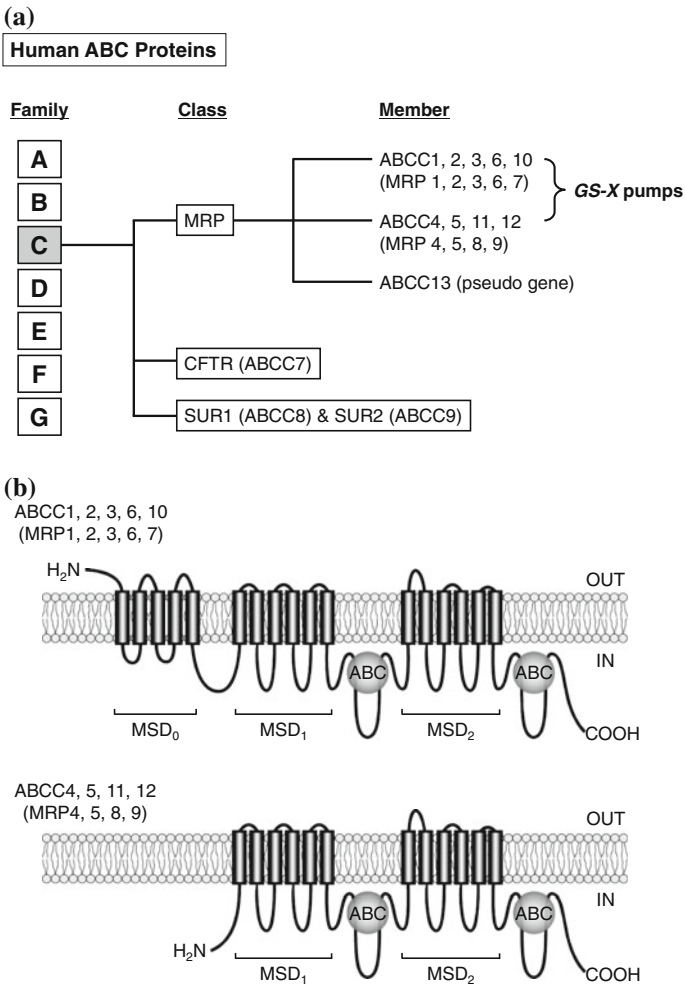

Fig. 5 a Schematic diagram representing the relationship among the multidrug resistance-associated protein (MRP) class members, cystic fibrosis transmembrane conductance regulator (CFTR), and sulfonylurea reseptors (SUR) in the human ABC protein gene C-family. **b** Schematic illustration of the structures of MRP class members. ABCC1, 2, 3, 6, 10 (MRP1, 2, 3, 6, and 7) have a total of 17 transmembrane regions (cylinders), clustered into three membrane-spanning domains (MSD_0, MSD_1, MSD_2), and two intracellular ABCs. ABCC4, 5, 11, and 12 (MRP4, 5, 8, and 9) have 12 transmembrane regions, as they lack MSD_0

putative mouse or rat orthologous gene corresponding to human *ABCC11* (Shimizu et al. 2003). This fact indicates that *ABCC11* is not an orthologous gene but rather a paralogous gene generated by gene duplication in the human genome (Shimizu et al. 2003).

When transfected exogenously, the ABCC11 protein was localized in the apical membrane of Madin–Darby canine kidney strain II cells (MDCK II cells) (Bortfeld et al. 2006). The substrate specificity of ABCC11 was characterized in more detail by an in vitro transport assay with plasma membrane vesicles prepared from pig LLC-PK1 cells transfected with an ABCC11 expression vector (Chen et al. 2005). The results of this assay demonstrated that ABCC11 WT is able to transport a variety of lipophilic anions including cyclic nucleotides, glutathione conjugates such as leukotriene C_4 (LTC_4) and S-(2,4-dinitrophenyl)-glutathione (DNP-SG), steroid sulfates such as estrone 3-sulfate ($E_1$3S) and dehydroepiandrosterone 3-sulfate (DHEAS), glucuronides such as estradiol 17-β-D-glucuronide ($E_2$17βG), the monoanionic bile acids glycocholate and taurocholate, as well as folic acid and its analog methotrexate (Bortfeld et al. 2006; Chen et al. 2005).

Impressively, a single nucleotide polymorphism (SNP) in the *ABCC11* gene was reported to determine the human earwax type (Yoshiura et al. 2006). Earwax (cerumen) is a secretory product of the ceruminous apocrine glands, which can be classified into two phenotypes in humans, wet (sticky) and dry. By using a positional cloning and linkage disequilibrium analysis of genetics of earwax type, Yoshiura et al. (2006) revealed that the nonsynonymous SNP (538G > A, Gly180Arg) in the *ABCC11* gene is the determinant of human earwax type. The A/A genotype gives the dry phenotype, whereas both G/A and G/G genotypes give the wet phenotype. This is consistent with observations that earwax type is a Mendelian trait and that the wet phenotype is dominant over the dry one. Loss of ABCC11 function is considered to be a cause for the dry phenotype. ABCC11 WT may function to secrete such "wet" components of earwax.

Functional studies with ABCC11-expressing LLC-PK1 cells demonstrated that cells expressing ABCC11 SNP with allele A (Gly180Arg) show a significantly lower level of cGMP transport, as compared with those expressing ABCC11 WT with allele G (Yoshiura et al. 2006). Gly180 is located in the first transmembrane helix of ABCC11 protein, and substitution of Gly180 to Arg180 gives a positive charge to the first transmembrane helix. This electrostatic change might affect the structure of this helix and thereby *N*-glycosylation of ABCC11 protein is impaired (Toyoda et al. 2009).

Toyoda et al. (2009) provided evidence that proteasomal degradation of the SNP variant (Arg180) of ABCC11 is the underlying molecular mechanism (Toyoda et al. 2009). ABCC11 WT with Gly180 is an *N*-linked glycosylated protein, which is localized within intracellular granules and large vacuoles as well as at the luminal membrane of secretory cells in the cerumen apocrine gland (Toyoda et al. 2009). *N*-linked glycosylation occurs at both Asn838 and Asn844 in the extracellular loop between transmembrane domains 7 (TM7) and 8 (TM8) of the ABCC11 WT protein (Toyoda et al. 2009). The *N*-linked glycans are thought to be subjected to extensive modification as glycoproteins mature and move through the endoplasmic reticulum (ER) via the *Golgi* apparatus to their final destinations as, for example, intracellular granules and large vacuoles of secretory cells in the apocrine gland. In contrast, the SNP variant (Arg180) lacks *N*-linked glycosylation and readily undergoes proteasomal degradation, most probably via ubiquitination. As a

consequence, no granular or vacuolar localization was detected in the cerumen apocrine glands of people homozygous for the SNP variant.

Immunohistochemical studies with cerumen gland-containing tissue specimens revealed that the ABCC11 WT protein with Gly180 was expressed in the cerumen gland (Toyoda et al. 2009). The cerumen gland is one of the apocrine glands. In addition to their presence in the external auditory canal, apocrine glands can be found in the axillary region and breast, whose physical characteristics also are concerned with apocrine glands. In fact, there is a positive association among the wet earwax type, axillary osmidrosis (Yoo et al. 2006), and colostrum secretion from the breast (Miura et al. 2007).

It is of great interest to note that the nonsynonymous SNP of 538 G > A (Gly180Arg) in the *ABCC11* gene exhibits wide ethnic differences in the allele frequency (Fig. 7). Among Mongoloid populations in Asia, the frequency of the 538 A allele is predominantly high, whereas its allele frequency is low among Caucasians and Africans. The frequency of the 538 A allele exhibits a east–west downward geographical gradient with the highest peak in northeast Asia. It is suggested that the A allele arose in northeast Asia (Yoshiura et al. 2006), apparently reflecting the intercontinental migration of *Homo sapiens* (Ishikawa et al. 2013a, b).

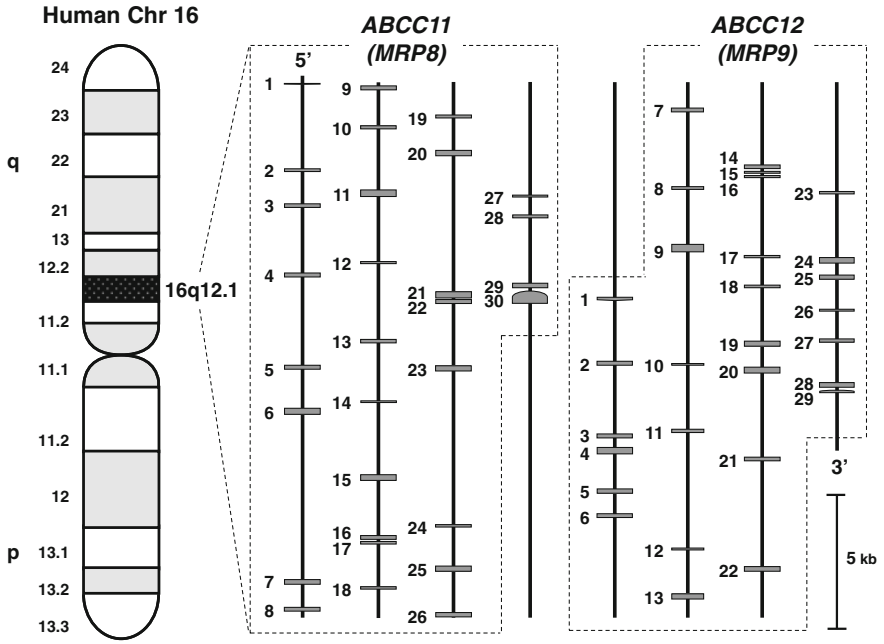

Fig. 6 **a** The genomic structures of *ABCC11* (*MRP8*) and *ABCC12* (*MRP9*) genes on human chromosome 16q12.1. The cytogenetic location of the *ABCC11* and *ABCC12* genes as well as the structures of exons and introns were analyzed by BLAST search on the human genome. The SNP 538G > A (Gly180Arg) resides on exon 4 of the *ABCC11* gene

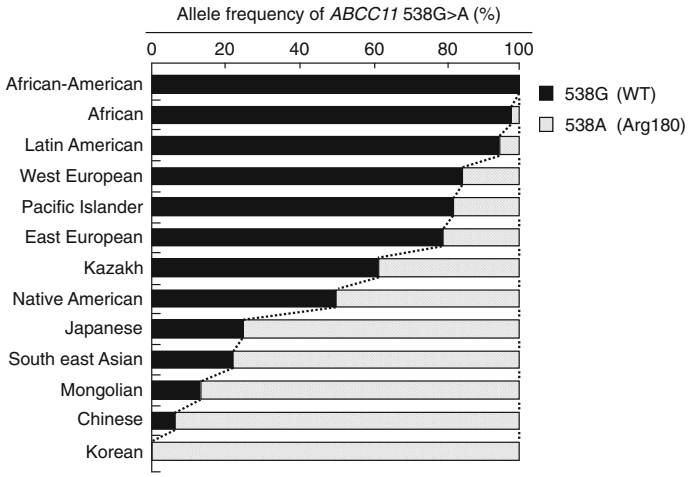

Fig. 7 The allele frequencies of the wild type (WT, Gly180) and the 538G. > A (Arg180) variant of human *ABCC11* (*MRP8*) gene among different ethnic populations. Data are from Yoshiura et al. (2006) and Ishikawa et al. (2013a, b)

Association Between SNP 538G > A (Gly180Arg) in *ABCC11* Gene and Axillary Osmidrosis

Recently, it has been demonstrated that the ABCC11 WT allele is intimately associated with axillary osmidrosis as well as the wet type of earwax (Table 2). Several studies have already concluded that the genotypes at *ABCC11* 538G > A would be useful biomarkers for the diagnosis of axillary osmidrosis (Martin et al. 2010; Inoue et al. 2010; Toyoda et al. 2009; Nakano et al. 2009). Therefore, it was expected that genotyping of the *ABCC11* gene would provide an accurate and practical criterion for guidance of appropriate treatment and psychological management of patients (Toyoda et al. 2009; Inoue et al. 2010; Ishikawa and Hayashizaki 2013).

Sweat produced by the axillary apocrine glands is odorless. Secretions from the apocrine glands, however, can be converted to odoriferous compounds by bacteria

Table 2 Association of *ABCC11* genotype with earwax type and axillary osmidrosis in Japanese subjects

Earwax type		Genotype at *ABCC11* 538G > A			Axillary osmidrosis patients
		G/G	G/A	A/A	
Dry	262	0	0	262	0
Wet	300	23	277	0	182

Data are from Ishikawa et al. (2013a, b)

(*Corynebacteria*), which results in the formation of the unique "human axillary odor" (Shehadeh and Kligman 1963). Axillary osmidrosis patients (538G/G homozygote or G/A heterozygote) were observed to have significantly more numerous and larger-sized axillary apocrine glands as compared with those in subjects carrying the A/A homozygote. Indeed, the 538G allele in the *ABCC11* gene is associated with axillary osmidrosis (Martin et al. 2010; Inoue et al. 2010; Toyoda et al. 2009; Nakano et al. 2009), and ABCC11 WT (Gly180) would be responsible for the secretion of preodoriferous compounds from the axillary apocrine gland. In primates, axillary odors may play a role in olfactory communication, although no documented behavioral or endocrine changes resulting from volatiles produced in the axillae have been reported to occur in humans. Previous studies have described the presence of androgen steroids in the axillary area. Androsterone sulfate (AS) and DHEAS were detected in an extract of axillary hairs in addition to high levels of cholesterol (Julesz 1968). It was also demonstrated, following injection of radioactive pregnenolone or progesterone, that steroid secretion was concentrated in the axillary area (Brooksbank 1970). In those studies, however, the axillary sweat collected from the skin surface contained a mixture of materials from apocrine, eccrine, and sebaceous glands, in addition to desquamating epidermal cells. In this respect, Labows et al. (1979) demonstrated that pure apocrine secretions contained at least two androgen steroids, AS and DHEAS, in addition to cholesterol. It is strongly suggested that one of the physiological functions of ABCC11 WT is the active transport of steroid metabolites, such as AS and DHEAS, into the lumen of apocrine glands.

Diagnosis of Axillary Osmidrosis by Genotyping *ABCC11* 538G

Today in Japan, axillary osmidrosis is recognized as a disease that is covered by the national health insurance system. Axillary osmidrosis, which is exemplified by unpleasant odors, sweating and staining of clothes, is often perceived, especially by young women, as a distressing and troublesome problem (Wu et al. 1994). Certain people display an excessive fear, aversion, or psychological hypersensitivity to unpleasant smells or odors. They tend to opt for aggressive surgical treatments and are sometimes categorized as having osmophobia. Interestingly, an association between wet-type earwax and axillary osmidrosis had already been recognized more than half a century ago (Matsunaga 1962). Hence, the wet type of earwax has frequently been used as one of the diagnostic criteria and characteristics in the clinic. This relationship, however, had only been based on the observations of those two respective phenotypes. Therefore, there has been a need for objective evidence for diagnosis of axillary osmidrosis to prevent unnecessary treatments for such patients.

The rapid growth of personalized medicine is being supported by emerging new technologies together with accumulating knowledge of pharmacogenomics. We have created a clinical method to genotype the SNP 538G > A in the human *ABCC11* gene (Ishikawa and Hayashizaki 2013). The clinical method based on isothermal DNA amplification reactions enables us to detect the genetic polymorphism in about 30 min under isothermal conditions. This method requires neither DNA isolation nor PCR process for sample preparation (Toyoda et al. 2009; Ishikawa and Hayashizaki 2013). To determine the SNP 538G > A (Gly180Arg) in the *ABCC11* gene, we prepared one set of primers designated TP, FP, BP, OP, and CP (Ishikawa & Hayashizaki 2013). The TPs discriminate the polymorphism 538G or 538A in the *ABCC11* gene, and the CPs inhibit the background amplification from mismatch sequence pairs (Toyoda et al. 2009; Ishikawa and Hayashizaki 2013). These primers selectively recognized the SNP 538G > A of the *ABCC11* gene to discriminate homozygous 538G/G, heterozygous 538G/A, and homozygous 538A/A. Genotype-specific reactions are monitored by measuring the fluorescence of SYBR®Green that is intercalated into amplified DNA products. The end-point determination can be achieved by introducing a CCD camera (Fig. 8), which enables simple and cost-effective detection. Thus, this genotyping method is expected to provide a practical tool for clinical diagnosis of axillary osmidrosis in Japan (Fig. 9).

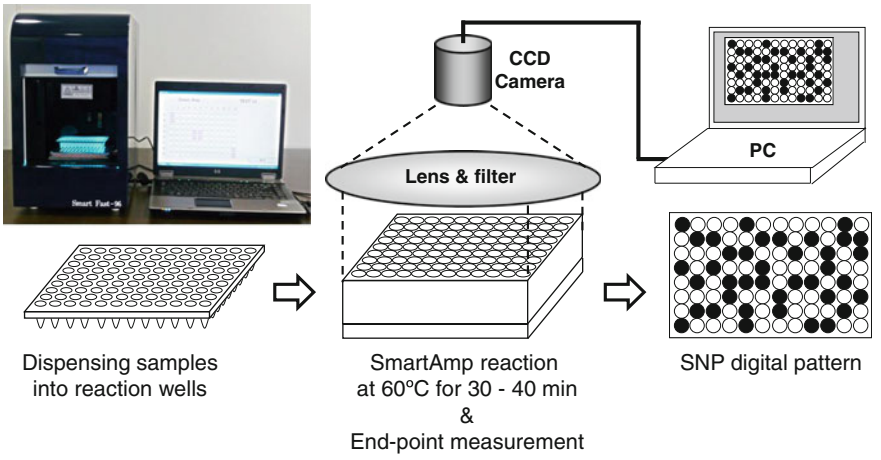

Fig. 8 Schematic illustration for end-point detection of SmartAmp-based SNP typing with a CCD camera-linked digital processor. Before being applied to the SmartAmp reaction, each blood sample is diluted 4-fold with 40 mM NaOH and then heated at 98 °C for 3 min. During this pretreatment process, proteins and RNA are denatured and degraded under alkaline conditions. After chilling on ice, 1 μl of the pretreated sample is added directly into the reaction mixture (final volume of 25 μl). To determine the SNP 538G > A (Gly180Arg) in the *ABCC11* gene, the reaction mixture contains one set of primers designated TP, FP, BP, OP, and CP (Ishikawa and Hayashizaki 2013)

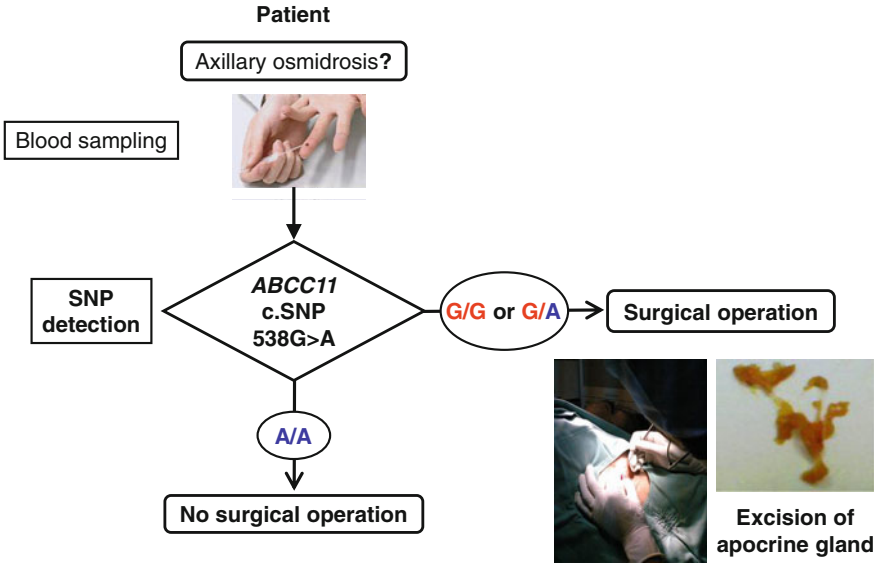

Fig. 9 Decision tree for the treatment of axillary osmidrosis. The genetic test of SNP in the *ABCC11* gene is one of the clinical factors that underlies doctor's decision. Patients carrying genotypes of 538G/G and 538G/A may be subjected to surgical operation of excising apocrine glands, whereas no surgical operation is needed for those who are carrying the genotype of 538A/A

Concluding Remarks

During the past three decades, remarkable advances were made in terms of analysis of the molecular structure and the genetics of human ABC transporters. Meanwhile, new technologies were also developed to accelerate the speed of basic research and clinical applications. The ABC transporters are a family of large proteins in membranes and are able to transport a variety of compounds including metabolites and drugs through membranes at the cost of ATP hydrolysis. Besides drug transport, the physiological function of ABC transporters includes the transport of lipids, bile salts, toxic compounds, and peptides for antigen presentation or other purposes, such as ion-channel regulation. The human genome contains 48 ABC transporter genes; at least 14 of human ABC transporter genes are reportedly associated with heritable human diseases that are rare and heavily transmitted in families. Mutations in ABC transporter genes have been reported to be associated with inherited diseases including Tangier disease T1 (*ABCA1*); Stargardt disease, retinitis pigmentosa, and age-related macular degeneration (*ABCA4*); progressive familial intrahepatic cholestasis (*ABCB11*); Dubin–Johnson syndrome (*ABCC2*); pseudoxanthoma elasticum (*ABCC6*); cystic fibrosis (*CFTR/ABCC7*); X-linked adrenoleukodystrophy (*ABCD1* and *ABCD2*); some forms of Zellweger syndrome (*ABCD3* and *ABCD2*), and sitosterolemia(a rare lipid metabolic disorder inherited

as an autosomal recessive trait) (*ABCG5* and *ABCG8*). Furthermore, it has recently been reported that SNPs in *ABCC11* and *ABCG2* genes are related to axillary osmidrosis and gout risk, respectively. Understanding the molecular mechanisms and clinical relevance of interindividual variability in drug response remains an important challenge (Ishikawa et al. 2013b). Pharmacogenomics, the study of genetic variation in the genes that influence drug effect, can provide insight into interindividual variability and a more accurate prediction of drug response than may be obtained by relying solely on a patient's clinical information. Membrane transporters have been increasingly recognized as contributing to variability in drug exposure and response and are important to evaluate during drug development and regulatory review to understand how relevant they are to benefit and risk of drugs. Therefore, the goal of drug transporter pharmacogenomics is to understand the impact of genetic variation on the function of transporters that interact with medications.

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ABCC7/CFTR

Robert C. Ford

Abstract The cystic fibrosis transmembrane conductance regulator (CFTR), also known as ABCC7, is an unusual member of the ATP-binding cassette family in that it is an ion channel rather than a transporter. Here, ATP binding is thought to initiate channel opening, with hydrolysis of the nucleotide being associated with channel closure. Loss of CFTR function through mutation leads to the life-threatening and -shortening condition known as cystic fibrosis. Recently, the importance of external factors affecting CFTR function has also been reported. In this chapter I have focussed on the basic structural biology and biochemistry of the protein but have also attempted to link this knowledge with the understanding of the disease. I have employed a bottom-up approach, starting with what can be learned from the primary structure of the protein, leading on to an examination of the secondary and tertiary structure of CFTR. The mapping of common CF-causing missense mutations within the CFTR 3D structure will also be addressed here. Lastly, the chapter finishes with some discussion of the prospects for future research on CFTR and how data for the channel function of CFTR can inform the ATP-binding cassette field of study in general.

Keywords CFTR · ABCC7 · Ion channel · Membrane protein · ATP-binding cassette · Cystic fibrosis

Abbreviations

ABC	ATP-binding cassette
C	Carboxy-terminal
CF	Cystic fibrosis
CFF	Cystic fibrosis foundation
CFTR	Cystic fibrosis transmembrane conductance regulator

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DDM	Dodecyl maltoside
EM	Electron microscopy
ECL	Extracellular loop
GPCR	G-protein coupled receptor
ICL	Intracytoplasmic loop
N	Amino-terminal
NBD	Nucleotide-binding domain
PDB	Protein data bank
PM	Plasma membrane
RI	Regulatory insertion
R	Regulatory (region or domain)
SCA	Statistical coupling analysis
TMD	Transmembrane domain

Introduction

As discussed at the end of this chapter, ABCC7 (hereafter termed CFTR) is one of the best-studied ABC transporters. It should be stated from the outset that, as far as we know, CFTR is not a transporter, but rather a channel allowing the flux of negatively charged anions such as chloride and bicarbonate. However, the fact that we know so much about it means that it sits quite comfortably in this book alongside more robust, active transporters that do a serious haulage job—transporting molecules out of, or into the cell. There is a wealth of data on CFTR and an abundance of excellent reviews available to the interested PhD student or researcher. This makes the job of doing justice to the breadth and depth of CFTR research well beyond the capacity of the author. Instead, the challenge of reviewing a relatively narrow window of CFTR research was chosen. In so doing there are hopefully some original insights in this chapter, or at least something to provoke the reader into a new direction of thought. As a structural biologist particularly interested in membrane proteins, the ABC transporter field is an ideal playground. With the exception of the GPCR family, the ABC transporter field is perhaps the most richly endowed with structural data for full-length transmembrane proteins. If one is allowed to count structures for the (mostly) water-soluble nucleotide-binding and substrate-binding domain structures of ABC transporters, then the ABC family dominates at the pinnacle of structural data for membrane proteins. This chapter will spend some time examining the primary structure (i.e. the amino acid sequence) of CFTR and some recent bioinformatics analyses. It is with good reason that we should start a review of CFTR with its primary structure: Not only do we have outstanding databases for CFTR missense mutations that set this ABC family member apart from its sibs, but also because in CFTR significant portions of its polypeptide chain are disordered. Hence primary structure is directly linked to function for these regions. The chapter then looks at the secondary and tertiary

structure of CFTR and finally tries to place the mutational information within the context of the tertiary structure.

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CFTR and Disease

Loss of CFTR channel function as a result of mutations in the *cftr* gene can cause the disease cystic fibrosis (Cant et al. 2014; Sosnay et al. 2013). This life-threatening and life-shortening condition affects mainly people of European origins, though why this population in particular is affected remains a mystery. Presumably at some point in human evolution, being a heterozygote with one mutated copy of the *cftr* gene carried a selective advantage. There have been many attempts to explain this, including theories around the past high mortality rates in children due to poor hygiene and intestinal infections. However no single explanation has yet been accepted by the majority of the cystic fibrosis (CF) research community (Cant et al. 2014).

CFTR dysfunction affects many organs in the body, but is arguably most serious in the lungs, where the lack of chloride efflux from the apical surface of epithelial airway cells causes shrinkage of the airway surface liquid layer and a rise in the viscosity of the mucus. The subsequently sticky mucus becomes the site of persistent bacterial and fungal infections, leading to inflammation and eventual remodelling and fibrosis of the airways (Riordan 2008). After many rounds of infection and inflammation, the lungs become more and more inefficient at oxygen exchange and have less capacity for inhaling air, leading to serious life-threatening disability. CFTR dysfunction has also been associated with other diseases where it is thought that CFTR activity is inhibited by external factors. For example, cigarette smoking has been associated with CFTR dysfunction (Clunes et al. 2012).

CFTR Primary Structure

CFTR (ABCC7) is a 1480-residue membrane protein, with the typical ABC transporter domain architecture of two transmembrane domains (TMDs), and two nucleotide-binding domains (NBDs) that are all fused in a single polypeptide chain

(Higgins 1992). This is described elsewhere in detail in this book and will not be re-worked here. Unique features to CFTR are an additional long (*circa* 200-residue) regulatory (R) region, as well as N- and C-terminal regions about 80 and 40 residues in length, respectively (Hunt et al. 2013). These regions are arranged in order from N- to C-terminus: N-term-TMD1–NBD1–R–TMD2–NBD2–C-term. It is important to recognise that having a long linker region between NBD1 and TMD2 is not unique to CFTR in the ABC transporter family. In plants, for example, the first member of the ABCA family also has a linker region between NBD1 and TMD2 that is over 150 residues long. However, it displays no significant sequence homology with the CFTR R region. Similarly, the ABCC family in mammals has several members with an additional transmembrane domain, often called the TMD0. This TMD of 3–5 transmembrane helices is joined to the standard TMD1 domain by a cytoplasmic linker that is also quite long (120 residues). From phylogenetic analysis, CFTR must have evolved from the ABCC subfamily, although there is little evidence to suggest any residual ABCC-type function in the present-day protein (Dean and Allikmets 2001). This 'C' family consists of several drug transporters with wide-ranging substrates. Glutathione transport is often associated with this family, and glutathione-conjugated drugs are common substrates.

Co-evolution of CFTR Residues

Given that CFTR has diverged from the ABC transporter family as a channel, then some insights into its channel function may be derived by bioinformatics analysis of its primary structure and the co-evolution of residues. A few attempts have been made to carry out such a bioinformatics analysis. A study of 2000 ABC transporter sequences that were extracted (by BLAST) on the basis of similarity to the CFTR sequence was carried out after filtering and processing by multiple sequence alignment. The data can be downloaded at http://cftrfolding.org/Mendoza_Thomas_Cell_2012/Mendoza_Thomas_Cell_2012.html (Mendoza et al. 2012). The resulting matrices show correlations between positions separated in the sequence but likely linked in terms of functional interactions or closely linked in terms of 3D space. The authors also employed the data to look for residues that could be mutated to rescue the effects of the most common disease-causing mutation in CF patients (F508 deletion).

Figure 1 gives an illustration of a subset of data from the 2D matrix employing one of the four statistical tests (statistical coupling analysis, SCA) (Lockless and Ranganathan 1999) used by the authors. Here, the numerical score provides a quantitation of the degree to which the probability of finding an individual residue at position i in the sequence is dependent on a given perturbation at a separate position j in the sequence. Correlation between three residues likely involved in CFTR channel properties in the sixth transmembrane helix of TMD1 (R334, I336, T338) and all other residues in the CFTR sequence is plotted. Regions of very low correlation (co-evolution) correspond to unique parts of the CFTR primary structure, as discussed above, as well as the C-terminal region which also shows little

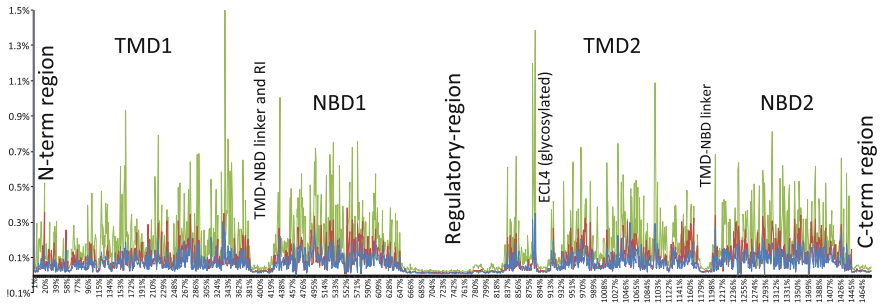

Fig. 1 Linear representation of the CFTR sequence with the major domains indicated above. The plot shows the SCA score for three selected residues in transmembrane helix 6 versus every other residue (see main text), (Mendoza et al. 2012). Key: R334 (*blue*), I336 (*red*), T338 (*green*). Regions with low scores correspond to regions that are likely to be specialised in CFTR channel and maturation functions, hence showing little or no conservation with other ABC proteins. Some of these regions are also likely to be structurally disordered. Interestingly the N-terminal region is thought to be disordered, but does not display scores close to zero hence may show more homology with other ABC proteins. The TMD–NBD linker regions are not disordered in available ABC protein exporter structures, but the CFTR linkers are quite different from other ABC proteins in terms of length as well as sequence (Hunt et al. 2013). In the first half of the protein, the TMD–NBD linker is closely followed by a disordered region in NBD1, the so-called regulatory insertion (RI)

similarity to any other ABC transporter. Interestingly, peaks in the SCA scores are distributed throughout the primary structure of CFTR and there are no immediately obvious clues as to the evolution of CFTR as a channel except in the regions of very low SCA score. Regions with low scores correspond to regions that are likely to be specialised in CFTR channel and maturation functions, and show little or no conservation with other ABC proteins. As mentioned above, a significant proportion of CFTR contains regions that are also likely to be structurally disordered and these all fall into low SCA score regions shown in Fig. 1. Interestingly the N-terminal region is an exception to this: It is thought to be disordered, but it does not display scores close to zero, implying more homology with other ABC proteins and that it may not be specialised for CFTR function. The opposite applies to the TMD–NBD linker regions, which are not disordered in the available high-resolution ABC protein exporter structures (where the TMD and NBD are fused). Here, the low SCA scores probably relate to the observation that CFTR linkers are quite different from those of other ABC proteins in terms of length as well as sequence (Hunt et al. 2013). This specialisation of the linkers in CFTR may be related to their location, which is along the ‘shoulders’ of the NBDs close to the inner leaflet of the membrane. Structural data for CFTR imply that this region may also be important as a location for the unique regulatory (R)—region of CFTR. Hence the linkers may have evolved to interact with the CFTR regulatory machinery and perhaps to act as a loose binding site for this otherwise disordered part of the CFTR structure. In the first half of the protein, the TMD–NBD linker region is closely followed by a disordered region in NBD1, the so-called regulatory

insertion (RI). This region seems to be specialised to CFTR, but deletion of the RI results in a NBD1 that behaves better in terms of solubility (Lewis et al. 2005; Protasevich et al. 2010).

Secondary and Tertiary CFTR Structure

To date, there is no high-resolution X-ray crystal structure of full-length CFTR nor for any other ABCC family member. However, electron crystallography methodology has been used to generate a Coulomb density map for CFTR at 9 Å resolution (or more correctly 1/9 $Å^{-1}$) (Rosenberg et al. 2011), and single-particle analysis has been used to solve a number of low-resolution structures (Mio et al. 2008; Zhang et al. 2009, 2010). The electron crystallography methodology has also revealed the structure of another ABCC family member (ABCC1/MRP1) (Rosenberg et al. 2010), and single-particle analysis combined with electron microscopy has provided structural data for the hetero-octameric ABCC8/SUR1 complex (Mikhailov et al. 2005). A number of computational atomic models of CFTR have been generated, based on similarity at the primary structure level with a bacterial homologue Sav1866 or the eukaryotic P-gp/ABCB1 (Mornon et al. 2008; Rahman et al. 2013; Serohijos et al. 2008). Structures of the isolated soluble domains of CFTR have also been reported, with the X-ray crystal structures of NBD1 (Lewis et al. 2004) and NBD2 (PDB: 3GD7, unpublished). Nuclear magnetic resonance (NMR) structures of NBD1 (Baker et al. 2007; Bozoky et al. 2013a, b; Hudson et al. 2012; Kanelis et al. 2010) and the (mostly disordered) isolated R region have also been reported (Baker et al. 2007).

The greatest detail in current CFTR structural knowledge comes from the X-ray structures of its NBDs, as reviewed recently (Hunt et al. 2013). Overall, the NBDs of CFTR have similar structural folds to those of other ABC proteins, including the common Walker A, Walker B and signature motifs and this is covered elsewhere in this book in detail (Lewis et al. 2004). The NBDs of CFTR also have a few unique features compared to other ABC proteins: Firstly, CFTR NBD1 has an additional ~35-residue regulatory insertion (RI) and the NBD1 structure probably includes a short extension that represents the first segment of the regulatory (R) region. Note that the atomic coordinates are available for NBD2 of CFTR (PDBID = 3GD7), however there is currently no publication describing the work behind the structural analysis. The NBDs of CFTR are not very symmetrical in terms of their primary structure, with less than 30 % identity between NBD1 and NBD2 sequences (Klein et al. 1999), compared to above 70 % identity in other ABC proteins (Higgins 1992). CFTR has changes in the signature sequence in NBD2 (LSGGQ to LSHGH) resulting in only one active ATP hydrolytic site, although both sites still have the ability to tightly bind ATP (Aleksandrov et al. 2002; Lewis et al. 2004; Thibodeau et al. 2005). Such strong NBD asymmetry is found with several other ABC proteins, e.g. MRP1/ABCC1 (Hou et al. 2000; Jones and George 2004). Structural and biophysical studies have suggested that the NBDs of CFTR form the same

ATP-dependent head-to-tail sandwich dimer conformation as other ABC proteins (Aleksandrov et al. 2009; Jones and George 2004; Mense et al. 2006; Rosenberg et al. 2011; Vergani et al. 2005).

The two TMDs of CFTR each consist of the usually encountered six transmembrane α-helices with three extracellular loops (ECLs) and two long intracellular loops (ICLs). The longest ECL is ECL4 in TMD2 which is also N-glycosylated (Chang et al. 2008). In contrast to other ABC transporters, the TMDs of CFTR are expected to form a continuous pore through which ions can pass passively (Gadsby et al. 2006; Riordan 2008). For other ABC transporters the TMDs form a binding site (or binding sites) for the transported substrates that can be alternately exposed to the outside or inside milieu via conformational changes (Jardetzky 1966). Comparison of the CFTR electron crystallography map to the X-ray crystal structure of a homologous bacterial ABC transporter Sav1866 has revealed a lozenge-shaped density between TM helices 3, 6, 9 and 12 that may account for the location of a channel pore-regulating gate (Rosenberg et al. 2011). This position correlates with the expected locations of biochemically identified pore-lining residues (Linsdell 2005; Smith et al. 2001) in CFTR structural models (Mornon et al. 2008; Serohijos et al. 2008). The pore appears to have a deep, wide vestibule on the extracellular side, which may be at least partially accessible via the outer leaflet of the lipid bilayer.

The ICLs are predicted to form part of the typical ABC protein coupling helices that form the interface between TMDs and NBDs (Serohijos et al. 2008). The isolated R region is a highly dynamic and disordered structure that is mostly predicted to be random coil and with around 5 % α-helical secondary structure, measured by NMR studies (Baker et al. 2007; Ostedgaard et al. 2000). NMR methods have also detected interactions between the R region and NBD1, NBD2 and the C-terminus when the R region is mixed with the other domain in vitro (Baker et al. 2007; Bozoky et al. 2013b; Kanelis et al. 2010). Electron microscopy studies have tentatively assigned at least part of the R region to a location on the 'shoulder' of one NBD and close to the lipid bilayer (Rosenberg et al. 2011; Zhang et al. 2010). The R region is highly charged and contains several target sites for protein kinase A (PKA) phosphorylation (Cheng et al. 1991; Csanady et al. 2005; Riordan et al. 1989), as well as being a substrate for other kinases (Chappe et al. 2003; French et al. 1995). Phosphorylation of the R region regulates CFTR channel function (Csanady et al. 2005; Dahan et al. 2001; Gregory et al. 1990; Seibert et al. 1999), but no single phosphorylation site seems to be indispensible. The disordered structure of the R region is likely to be important to maximise accessibility of kinases for phosphorylation (Chong et al. 2013), however how phosphorylation of a region that is disordered then goes on to promote the activity of the channel is not well understood.

Electron microscopy studies of full-length CFTR showed that the protein is homologous to X-ray crystal structures of other ABC exporters, in particular Sav1866 (Dawson and Locher 2006). Such data have also shown that CFTR can display an outward-facing conformation in the *absence* of ATP (Rosenberg et al. 2004, 2011; Zhang et al. 2009, 2010). This is somewhat at odds with the idea that

the inward- and outward-facing conformations of CFTR represent the closed and open channel configurations, respectively (Vergani et al. 2005) and (Wang and Linsdell 2012). At first sight, therefore, the structural data seem to indicate a structural miscoupling in CFTR between ATP binding and hydrolysis and switching between outward-facing and inward-facing conformations, respectively. However, other structural studies of ABC transporters have also revealed a slightly less than obligatory coupling between nucleotide status and conformation. For example, inward-facing conformations of the mitochondrial ABC transporter ABCB10 have been observed in the presence of nucleotide (Shintre et al. 2013). Similarly, outward-facing conformations in a pretranslocation state have been reported for a nucleotide-free state of the maltose ABC transporter MalFGK (Oldham et al. 2013).

The quaternary structure of CFTR has also been inferred from biophysical data. CFTR monomers were crystallised in two-dimensional arrays that were two molecular layers thick (Rosenberg et al. 2011). Conversely, single-particle analysis revealed dimeric CFTR particles (Awayn et al. 2005; Mio et al. 2008; Zhang et al. 2009) in the same detergent, whilst more recent studies of CFTR in novel facial detergent showed a monomeric state (Hildebrandt et al. 2014). It is important to note that all these structures were obtained for CFTR in different detergent micelles and may not reflect the oligomeric status of the protein in a biological membrane. The quaternary structure of CFTR in vivo still remains a matter of debate, especially as both monomers (Chen et al. 2002; Haggie and Verkman 2008; Ramjeesingh et al. 2001) and dimers (Eskandari et al. 1998; Ramjeesingh et al. 2001; Schillers et al. 2004) have been proposed for CFTR that was integrated into a lipid membrane environment.

CFTR Tertiary Structure Explored by Electron Crystallography

The highest resolution experimentally determined structure for the full-length CFTR protein has been derived from electron crystallography of two-dimensional crystals. These are crystals that are a single or few molecules thick, hence are very fragile and must be supported on a flat surface provided by the carbon film of an electron microscopy grid. The spatial resolution of the CFTR map derived from these crystals was reported to be at about 1/9 $Å^{-1}$ (i.e. densities about 9 Å apart can just be resolved from each other). The thickness of the crystals (circa 300 Å, composed of two CFTR molecular layers) as well as the low symmetry (C1 symmetry) required a lengthy and painstaking data collection and processing. Nevertheless, at this level of resolution (1/9 $Å^{-1}$), domains should be readily identifiable and some long transmembrane helices should also be distinguishable (especially if they are well separated from the main helical bundle). For reference, a theoretical study using the ABC transporter BtuCD (Ford and Holzenburg 2008) showed that even with the usual signal: noise and missing cone defects in electron

crystallography data, it should be possible to resolve transmembrane helices at about 1/8 $Å^{-1}$ and that at 1/10 $Å^{-1}$ well-separated and long helices in the BtuCD structure could still be discerned. On the other hand, beta strands and short helices in the NBD region could not be discerned—even at 1/8 $Å^{-1}$ resolution.

A good example of the restriction of resolution on the interpretation of the CFTR map is shown in Fig. 2. Elongated segments of density from the experimental CFTR map can be fitted with transmembrane alpha helices. In this case the helices are shown well separated from surrounding densities. Interpretation of the map using a published homology model for CFTR (Mornon et al. 2009, 2014) assigns the longest helical density to the seventh transmembrane helix, residues 860–891. This helix and the nearby transmembrane helix 8 could be more confidently assigned because they extend much further on the extracellular side of the protein than the other transmembrane helices. The large extracellular loop that links these

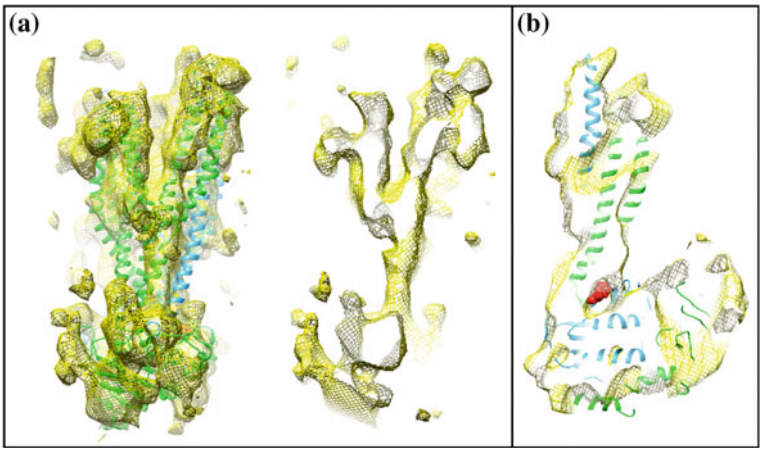

Fig. 2 **a** Experimental outward-facing CFTR structure derived from electron crystallography (*yellow mesh*, *left*) with gaping TMDs at the top and closely contacting NBDs at the bottom. The map is at a resolution where long and well-separated transmembrane helices can be distinguished, as shown in the 1-nm thick slice through the map (*right*). CFTR closely resembles other structures for ABC transporters in the outward-facing state, and the map can be interpreted using a homology model based on the Sav1866 transporter (Dawson and Locher 2006; Mornon et al. 2009) shown in the *left*. The *blue ribbon trace* is from the N-terminal half of the model, *green* trace is from the C-terminal half. Potential flexible regions (N-terminal 80 residues, R region, RI region) were removed from the model before fitting to the map. The final correlation coefficient between the CFTR map and a suitably resolution-adjusted map calculated from the model was about 0.65 (where 1.0 would be perfect correspondence, and Sav1866 fitting gave 0.56), reflecting the systematic deviation from the symmetric Sav1866 structure during evolutionary timescales. Models can be further refined with some local flexible fitting (Senderowitz, Bar-Ilan University, unpublished data) with a correlation coefficient approaching 0.9. **b** Illustration of the pivotal position occupied by F508 (*red space-filling atoms*) in the model. It sits at the interface between ICL4 in TMD2 (*green ribbon*) and NBD1 (*blue ribbon, bottom*). The position of F508 in the model corresponds to a small cavity in the experimental map, again pointing to the local discrepancies between the Sav1866-based homology model and CFTR, as discussed below

two transmembrane helices (ECL4) is the site of glycosylation in CFTR (arrows, centre panel, Fig. 2, see also Fig. 1). In contrast, short helices, closely packed helices and beta sheets are not easily distinguishable at this resolution (see Fig. 2, all panels, for examples of this).

CFTR Mutations

To date, there are over 2000 different CFTR mutations that are logged as CF-causing. However, a high proportion of CF patients have protein with deletion of phenylalanine at position 508 (F508del) on at least one chromosome (Cystic Fibrosis Mutations Database, available at http://www.genet.sickkids.on.ca/cftr/). All heterozygotes with one WT copy of the gene display no disease (Cutting 2005; Riordan et al. 1989). CFTR mutations are functionally classified into several groups (Prickett and Jain 2013): Class 1 mutations cause a defect in CFTR protein synthesis, such as the premature stop codon W1282X, that results in little or no CFTR at the plasma membrane (PM). Class 2 mutations, including the common F508del, are translated into full-length nascent polypeptide chains but are defective in folding and are thus targeted for degradation rather than trafficked to the PM. Class 3 mutants of CFTR are able to reach the PM but have channel gating defects that decrease channel open time and decrease chloride flux, e.g. the second most commonly encountered mutation, G551D. Class 4 mutants reach the PM, but with decreased channel conductance. Class 5 mutants represent a fully functional CFTR at the PM but with reduced levels due to defective mRNA splicing. Classes 1–3 are associated with more severe disease phenotypes, whereas classes 4 and 5 are thought to be milder disease-causing mutations. It should be noted that some CFTR mutations may have more than one effect, for example the F508del mutation has reduced channel activity and shorter PM half-life in addition to the maturation and processing defects (Hwang et al. 1997).

As the vast majority of patients harbour F508del CFTR, this mutation has received the most attention. The ΔF508 mutation destabilises CFTR structure and folding (Lewis et al. 2005) and during protein synthesis, most of the expressed F508del CFTR is targeted by the endoplasmic-reticulum-associated protein degradation (ERAD) pathway for degradation by the proteasome, with only 1 % of translated protein reaching the PM (Kopito 1999; Ward et al. 1995). The small amount of ΔF508-CFTR that reaches the PM is a functional channel, although operating with slightly altered gating, namely with more time spent in the closed state (Cui et al. 2006). Significant levels of misfolding might cause gross changes in the overall F508del-CFTR structure, which could be detected by e.g. increased susceptibility to limited proteolysis (Hoelen et al. 2010; Peters et al. 2011), see also (Zhang et al. 1998). Surprisingly, however, the X-ray crystal structures of isolated F508del-NBD1 showed little structural differences compared to wild-type CFTR (Lewis et al. 2005, 2010). This has led to the hypothesis that F508 is important in inter-domain stabilisation and assembly, rather than the folding of NBD1 itself

(Lewis et al. 2005, 2010; Loo et al. 2010; Rabeh et al. 2012; Serohijos et al. 2008; Thibodeau et al. 2005). The F508 residue lies on the surface of the NBD1 in an area that, in the X-ray crystal structures of other ABC proteins, forms crucial interactions with the second intracytoplasmic loop of the opposing TMD region (Aller et al. 2009; Dawson and Locher 2006). Hence the F508-mediated interaction in CFTR is between NBD1 and ICL4 of TMD2 (Cui et al. 2007; Lewis et al. 2010; Loo et al. 2010; Rabeh et al. 2012; Serohijos et al. 2008; Thibodeau et al. 2005). It has also been suggested that the peptide backbone at position 508 is important for CFTR folding but that the phenyalanine side chain is necessary for inter-domain contacts and stability of the folded protein (Thibodeau et al. 2005).

As well as the destabilisation of NBD1–TMD2 interactions, F508del-NBD1 itself appears to be globally destabilised. Recombinant isolated F508del-NBD1 has a thermal unfolding transition about 6 °C lower than its wild-type counterpart (Protasevich et al. 2010). The F508del mutation is proposed to thermodynamically favour the formation of a molten-globule state that is prone to aggregation (Wang et al. 2010). The thermal destabilisation effect of the F508del mutation is also inferred from in vivo experiments showing that low temperature growth conditions are able to rescue F508del CFTR allowing it to progress to the PM (Denning et al. 1992). Molecules that can bind and thermostabilise F508del NBD1 in vitro have also been effective in correcting full-length F508del CFTR in cells (Sampson et al. 2011). However, there is some controversy over whether F508del CFTR can be rescued by stabilising NBD1 alone (Aleksandrov et al. 2012), or whether the correction of NBD1–TMD2 interactions is also required (Rabeh et al. 2012). A combination of both may be required.

Mapping of Common CF-Causing Missense Mutations Within the CFTR Structure

The large majority of mutations catalogued in the CF mutation database have not been well characterised in terms of their functional effects on the protein, and the rarer mutations inevitably occur in combination with a more commonly occurring mutation on the other copy of the *cftr* gene. In response to this deficit in our knowledge, the most commonly encountered mutations in CF patients have been included in a study of their effects at the cellular level—the so-called CFTR2 study (http://www.cftr2.org/). The common missense mutations in this study can be readily mapped on to the existing CFTR homology models and within the electron crystallography-derived Coulomb density map as shown in Fig. 2, hence it is possible for the first time to begin to correlate the functional effects of mutations with their likely structural context.

F508del is the most common disease-causing mutation by far. Does the CFTR structure explain why the loss of this single residue causes such a major temperature-dependent defect on the protein? Panel b in Fig. 2 shows the location of F508 in the density map (arrow). This view emphasises the importance of the F508

residue in mediating interactions between the first NBD (blue) and the second TMD, in particular ICL4 (green). Loss of the F508 residue would remove a contact point between the domains, which lies in a narrow bridge of density with little surrounding density. Removal of this bridging residue would be predicted to destabilise the tertiary structure of CFTR to a greater extent than its local effects on the stability of the isolated NBD1 (Protasevich et al. 2010). It is also interesting to note that the expected position of F508 in the CFTR density map corresponds approximately with a small cavity in the map that would not be expected to be there from the homology model. Such density voids in experimental maps may arise for the obvious reason—that this part of the map is filled with solvent rather than protein. However, voids can also occur if there are variable conformations for the protein in this particular region. This arises because the map is derived from the summation of data from many millions of molecules. Hence a region where the protein contributes density but where each molecule has a slightly different configuration could result in a lower (but not zero) density. Since maps such as the one shown in Fig. 2 are generally 'binary' in the sense that a given density threshold is chosen as a cut-off for displaying the map, such subtleties in the map can often be lost and a solvent-filled void may not be distinguishable from a region of disorder. On the other hand, use of a low density threshold runs the risk of including noisy features in the interpretation of the map. Tentative inspection of the map in this region with a lower density threshold implies that there is still a small cavity formed between F508 and residues that would contribute density in this region from ICL4 of CFTR, hence arguing for better homology models as well as better experimental data.

Of the well-characterised missense mutations listed in the CFTR2 database, there are more disease-causing mutations in the first half of the protein and especially in NBD1, but none in the linking regulatory region. It would seem reasonable that missense mutations in the less ordered R region could be tolerated with little impact on the functioning of the protein. Perhaps unsurprisingly, there is a clustering of disease-causing missense mutations in the three-dimensional structure, which may provide insights into the likely consequences of these mutations as well as into the most crucial regions of CFTR for its function. For example, if just the 30 most common disease-causing missense mutations in humans are considered, then two discrete clusters become obvious (Fig. 3a, b): One cluster, mostly in NBD1, probably relates to the previously discussed sensitive position of the ICL4/NBD1 interface where F508 is positioned. However, the homology model based on a bacterial transporter may not completely reflect the arrangement of residues in the CFTR structure, hence it is useful to superimpose the experimental map for CFTR on to the model. For example, Fig. 3c displays the map around the cluster of missense mutations close to F508, which is composed of F508, I507, V520, A559 and R560 in NBD1 and R1066, L1065 and L1077 in ICL4. As mentioned above, F508 sits close to a cavity in the map, suggesting contacts in this region between NBD1 and ICL4 in CFTR are much less robust than in the Sav1866 structure (Dawson and Locher 2006; Mornon et al. 2009, 2014).

A second cluster of more common missense mutations causing the CF disease is found mainly in TMD1 and appears to relate to pore-lining or gating residues that

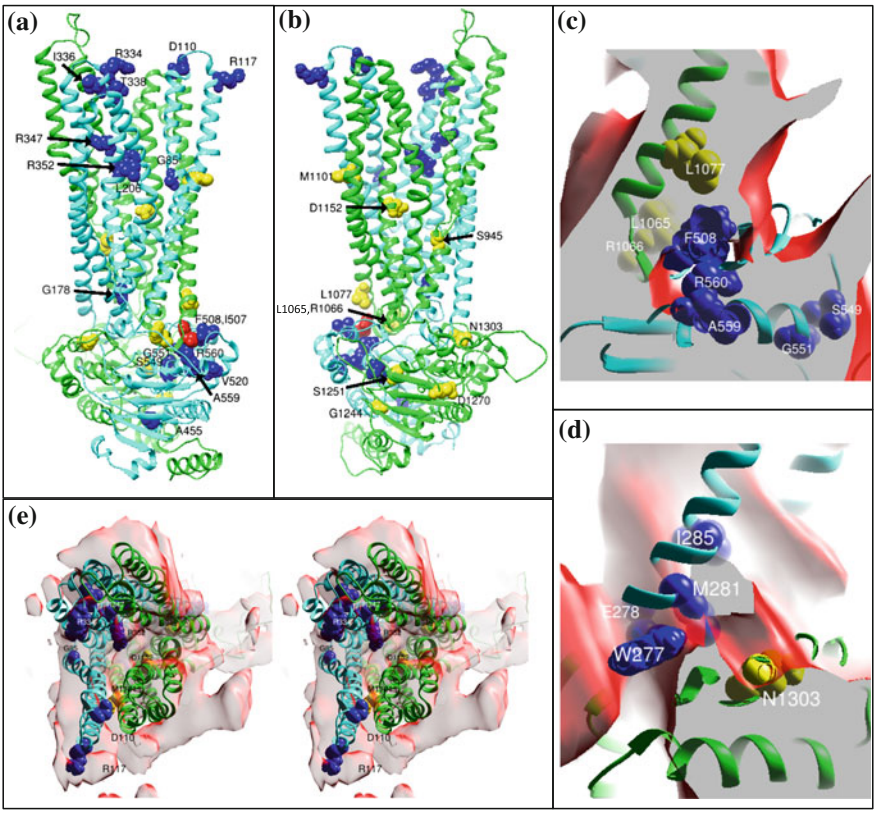

Fig. 3 **a** Location of residues in the N-terminal half of CFTR that, when mutated to other residues, causes CF. Of the 20 most commonly encountered mutations, 18 are displayed. P67 and R74 are N-terminal residues missing from the fitting, but present in the originally published model, and probably reside in the 'elbow' helix that lies parallel to the surface of the cytoplasmic side of the membrane. Residues in the first half of CFTR are coloured *light blue* and disease-causing mutated residues in *space filling/dark blue*. **b** Location of residues in the C-terminal half of CFTR that when mutated causes disease. The 10 most commonly encountered are shown as space-filling representations in *yellow*. **c** Zoom into the cluster of residues related to disease at the interface between NBD1 and ECL4 in TMD2 close to F508 (*space filling*) within the context of the experimental map (*red surface with slice plane transparent grey*). Note the small cavity in the map in the region of the F508 residue that is discussed in the text. **d** Focus on residues associated with disease-causing missense mutations in ICL2 in TMD1 that are close to N1303 in NBD2. **e** Stereo image looking down the central pore formed by the TMDs and from the extracellular side, with residues associated with disease-causing mutations shown as space-filling representations. A cluster of residues associated with channel gating dysfunction (when mutated) is present *top left*. The experimental map shows a cleft on the *right-hand side* that is not present on the *left*

follow a path from one side of the membrane to the other. This cluster is composed of R334, I336 and T338 on the extracellular side and L206, R347, R352 towards the centre of the membrane whilst D1152 in TMD2 is further on the cytoplasmic side of the membrane. A stereo image is included to be able to visualise this

pathway through the CFTR channel (Fig. 3e). Note that the majority of the disease-causing mutations are on the left-hand side of the figure and cluster in TMD1 (blue residues). Few commonly occurring missense mutations appear on the right (TMD2, yellow residues). The experimental map shows a more significant deviation from twofold symmetry than the homology model (which is biased by the twofold symmetry of the Sav1866 transporter used as the model). The most obvious manifestation of this asymmetry is the lack of a pronounced gap on the left-hand side of the protein. In the Sav1866 structure, gaps communicating to the extracellular leaflet of the lipid bilayer exist on both sides of the transporter and these gaps have been reported for other ABC transporters (Ward et al. 2007). This asymmetry in the CFTR structure may be a reflection of the evolution from a transporter to a channel, and may explain why common disease-causing mutations cluster on one side of the transmembrane region rather on the side that resembles more closely the Sav1866 transporter.

Is there a structural and functional equivalent of F508 in NBD2 or is the asymmetry in CFTR sufficient to distinguish between the interaction sites at NBD1/ICL4 versus NBD2/ICL2? The most obvious analogue for F508 in NBD2 is an asparagine residue, N1303. Mutation of this residue to lysine (N1303 K) is the third most common missense mutation, present in 1200 patients worldwide, or about 1.5–2 % of alleles. Like F508del, N1303K leads to failure of maturation of the mutated protein (Sosnay et al. 2011). N1303 deletion does not appear in the CF mutation database, however. There are several residues in ICL2 of TMD1 that are predicted to pack closely with N1303 and these also result in disease when mutated or deleted. Methionine 281 is most closely associated with N1303 in the modelling (Fig. 3d). The M281T mutation has been associated with pancreatitis (de Cid et al. 2010), but because of its rarity, is not one of the mutations studied so far in the first sweep of the CFTR2 study (Sosnay et al. 2013). Nearby residues in ICL2 have also been reported as CF-causing (W277R, I285F) and their locations with respect to N1303 are shown in Fig. 3d. A deletion of a residue in this region of CFTR can also be disease-causing (E278del), although in this case the deleted residue is found in ICL2 rather than in NBD2 and a glutamate residue is much less bulky than phenylalanine. These ICL2/NBD2 interface residues lie in regions that show a significant co-evolution in ABC proteins (Mendoza et al. 2012) as shown in Figs. 1 and 4 panels a and c. Interestingly, two of the main peaks in the co-evolution scoring for N1303 correspond to residues that become disease-causing when mutated to other residues (e.g. W277R, M281T—but not S269). Worthy of mention is the striking period of 4 oscillation that is displayed when several residues flanking N1303 are included in the correlation plots versus ICL2. This probably arises because of the helical secondary structure flanking ICL2 and reflects the likelihood that every fourth residue in a helix will occupy the same position, approximately, along the length of the helix.

Similar co-evolution scoring patterns exist for F508 and ICL4 residues (see panel b, Fig. 4). In this case, the main peak in the scoring corresponds to L1065 (can be mutated to F/R/P) and R1066 (can be mutated to C/S/H/L). Both of these residues when mutated can cause CF. The second noticeable peak corresponds to

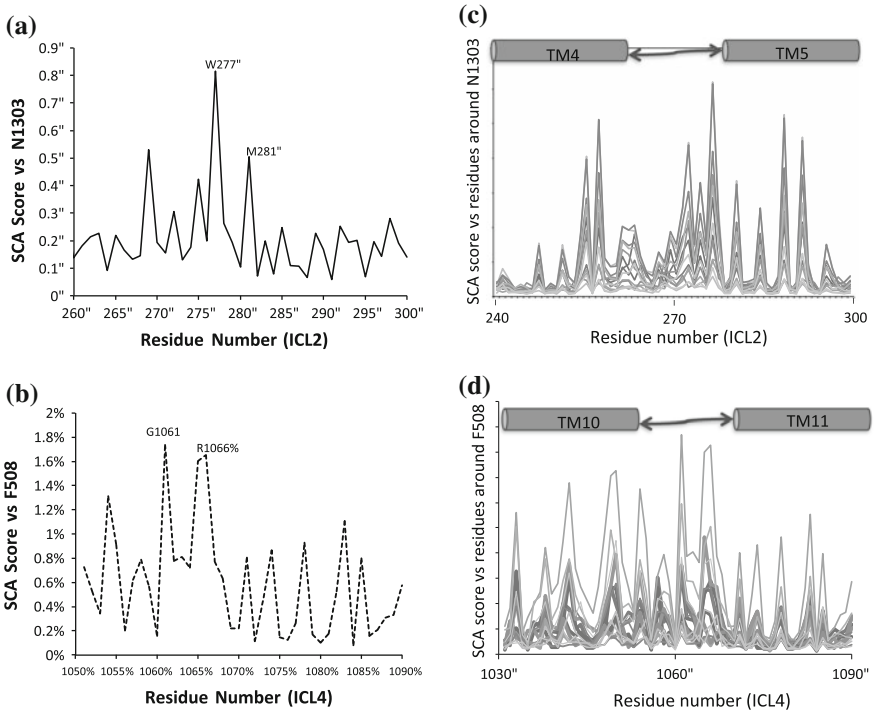

Fig. 4 Co-evolution of residues in ICL2 versus N1303 in NBD2 (**panel a**) and in ICL4 versus F508 in NBD1 (**panel c**). Mutations of W277 and M281 in ICL2 are CF-causing (see main text). **Panel c** Correlation scores for several residues in the vicinity of N1303 versus residues in ICL2. A period of 4 oscillation is apparent in the SCA scores (*cylinders*) that is probably reflecting the helical secondary structure flanking the linking non-helical connecting loop (*double headed arrow*). A similar profile is observed for residues in ICL4 versus residues around F508 in NBD1 (**panels b, d**), although with a less clear-cut pattern, perhaps reflecting the relative looseness of this interface as discussed in the main text. Data extracted from the database is described in (Mendoza et al. 2012)

G1061 which is also disease-causing when mutated to arginine. Surprisingly, R1070 does not score highly, despite it being a common disease-causing locus. For example, R1070 W is disease-causing on its own, but when in combination with F508del, W at position 1070 can alleviate the effects of the nearby phenylalanine loss. The period of 4 oscillation in the correlation with ICL4 residues for corresponding residues around F508 del is also apparent in panel d of Fig. 4, but the oscillation is noticeably weaker. This may be a reflection of deviation of a strict helical secondary structure in this region of the protein. It is also possible that this is a reflection of the weaker density in this region of the experimental CFTR density map (Figs. 2 and 3) and the apparent void close to F508 and ICL4. It has been speculated that this region of CFTR is the 'Achilles Heel' of the CFTR protein (Rosenberg et al. 2011).

Conclusions

CFTR is one of the best-studied ABC transporters (but with the caveat that it is not a transporter). The intense research efforts on CFTR are being driven in response to the devastating effects of mutations in the CFTR gene and their relative frequency in one part of the human population. Fortunately, these large-scale efforts into understanding CFTR have been generously funded by charities such as the US—based Cystic Fibrosis Foundation. Encouragingly, there are now some new drugs that have proven to be very effective at treating the basic defect in the disease—and at least for some mutations and patients, a cure seems very likely (Sosnay et al. 2013; Van Goor et al. 2009, 2011).

It could be argued that CFTR can give us a unique insight into structure–function relationships for ABC transporters and so merits a place in this book despite its lack of transport activity. The huge database that exists for CF-causing mutations is invaluable in the ABC transporter field,—see http://www.genet.sickkids.on.ca—and the work of the CFTR2 consortium will build on that knowledge with more direct functional data for disease-causing mutations (Sosnay et al. 2013). We should also not forget that, uniquely in the ABC transporter field, the activity of CFTR could be monitored at the single molecule level and with very high temporal resolution (Aleksandrov et al. 2007; Hwang and Sheppard 2009). This has allowed a detailed dissection of some aspects of its function that are the envy of researchers working on other ABC transporters.

In some other areas, however, progress in CFTR research has been very difficult compared to other ABC proteins. Expression levels of CFTR in normal epithelial cells are quite low. The most enriched source of CFTR has been reported to be from the rectal gland of sharks (Riordan et al. 1994) and, perhaps not too surprisingly, isolation of the protein from naturally expressing cells has been a highly specialized activity. Overexpression of the protein in stably- or transiently transformed cells has been the main approach for the subsequent physical isolation of the protein. In these cell types a high proportion of even the wild-type protein is degraded before it can reach the plasma membrane, and the large flexible regions of CFTR such as the regulatory region (see Fig. 1) are prime targets for intrinsic proteases. The inherent instability of the protein is compounded by a lack of solubility in many detergents. Some detergents based on lysolipids have been reported to efficiently solubilise the protein (Huang et al. 1998; Matar-Merheb et al. 2011; Pollock et al. 2014), but these may be too harsh for the full preservation of CFTR activity (Matar-Merheb et al. 2011; Pollock et al. 2014).

As a result of the difficulties in biochemical isolation, only a few studies have been carried out on the (relatively modest) ATPase activity of purified CFTR and our understanding of how it correlates with channel opening is still sketchy. Unlike other ABC transporters, there is no substrate-induced stimulation of ATPase activity in CFTR that would allow one to distinguish its activity from contaminating ATPases. There is some evidence that phosphorylation of the protein by protein kinase A (PKA) stimulates the ATPase activity of CFTR, and this would make

some sense in terms of the prerequisite of phosphorylation for channel opening (and subsequent closing) (Eckford et al. 2012). However PKA is, itself, a good ATPase and hence must be removed from CFTR before the latter protein's activity can be assayed. This adds to the difficulty of the assay. Like many other ABC transporters, the ATPase activity of CFTR preparations is strongly influenced by the detergent employed in the purification scheme and depends on whether the protein is reconstituted into proteoliposomes or not (Pollock et al. 2014). Despite these difficulties, there has been significant progress over the last few years. New yeast expression systems for the protein have been reported to yield milligram quantities of the purified protein (Pollock et al. 2014) and these reagents have been made available to the general CF research community. Technology for mammalian cell expression continues to improve, making it possible to use these systems for production of significant quantities of the protein. Thus it seems promising that CFTR biochemistry will soon become a standard approach in many laboratories around the world, leading to a much better understanding of its function and of the evolution of the ABC transporter family in general.

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Inborn Errors of the Cellular Expression and Localization of ABCG2 and ABCB6. A Database for ABC Transporter Mutations

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Abstract This chapter gives an overview on the cellular expression and function of ABCG2/BCRP/MXR and ABCB6. Inborn errors of ABCG2 are implicated in cancer multidrug resistance, hematological diseases and gout, while those of ABCB6 cause rare and poorly defined conditions, affecting eye development or pigmentation. We discuss the basic biochemical, physiological, and pathophysiological properties of these transporters, focusing on polymorphisms and mutations that lead to pathological conditions. Since in several cases the related diseases are caused by aberrant protein folding, trafficking or degradation, we describe potential correction strategies for prevention or treatment. In this chapter we also provide an improved database for the analysis of disease-causing mutations in ABC transporters, with the hope of promoting further basic research and clinical studies.

Keywords ABCB6 · ABCG2/BCRP/MXR/ · Cellular localization · Trafficking · Functional correction · Mutational database

Introduction

Human ABC transporters may form channels, active transporters and may also act as regulators, thus are involved in a wide range of physiological functions. A large group of human ABC transporters are active, ATP-dependent exporters of endo- and xenobiotics, and protect our body against the accumulation of harmful

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chemicals or various drugs. In this chapter we focus on two so-called "ABC half-transporters" both of which form homodimers in order to become functional. Their exact function may not be fully understood, but problems of their cellular localization and processing were shown to result in various human diseases. Interestingly, reduced expression and function of both ABCG2 and ABCB6 is relatively frequent and was thought to be asymptomatic. Still, during stress conditions, metabolic alterations, food or drug exposures the function of these proteins becomes clearly important. Under such conditions even relatively frequent polymorphisms may play a decisive role in disease development. An interesting, recently realized connection between these two ABC transporters is their relatively high level expression in the red cell membrane, and their contribution to the human blood group antigen repertoire. We document the potential utilization of this feature in analyzing transporter regulation and promoting diagnostics. Since a better understanding of the biochemical and physiological consequences of mutations in clinically relevant human ABC transporters may lead to potential cures or disease prevention, here we also provide a significantly extended and improved database for the analysis of disease-causing ABC protein mutations.

Diseases Connected to the Expression of Too Much or Too Little of ABCG2 (BCRP/MXR)

Structure, Transport Properties, Substrates, and Localization of ABCG2

The ABCG2 protein is a "half ABC transporter", containing one TMD with six transmembrane helices and one NBD, while the functional form of ABCG2 is a homodimer or homo-oligomer (Ozvegy et al. 2001; Gottesman et al. 2002; Dean et al. 2005; Sarkadi et al. 2006). ABCG2 resides in the plasma membrane and in polarized cells in the apical (luminal) membranes. As in the case of all eukaryotic ABC transporters working as "pumps", this protein is an ATP-dependent exporter. ABCG2 is a multidrug-type transporter, and thus has a promiscuous capacity of recognizing and extruding a large number of transported substrates—including many hydrophobic toxic compounds, amphiphilic positively or negatively charged agents, as well as practically water-soluble molecules. This protein is a physiologically important member of the xenobiotic defense systems in our body, and a major player in cancer drug resistance (Sarkadi et al. 2006). Interestingly, human ABCG2 is also an efficient uric acid transporter, and mutations or polymorphic variants of this transporter are causative in the development of gout (Woodward et al. 2009). ABCG2 may also be transporting heme and its metabolites (Krishnamurthy et al. 2004).

In the human body ABCG2 is expressed in many cell types and tissues. High level expression can be observed in the major metabolic and toxin elimination

centers, the liver (bile canalicular membranes) and the kidney (proximal tubular luminal membranes). Other key sites for ABCG2 expression are the barrier forming tissues, including the lumenal side of intestinal epithelial cells, the amnion epithelial cells and the chorion trophoblasts of the placenta, and the endothelial cells of the blood–brain barrier. ABCG2 is preferentially expressed in both pluripotent and some multipotent stem cells, e.g., in the hematopoietic progenitors, the proliferating keratinocytes of the epidermis, or the stem cells of limbal epithelia (Dean et al. 2005; Watanabe et al. 2004). In fact, ABCG2, causing a side-population phenotype of a Hoechst dye uptake, is considered as a marker for both normal and cancer stem cells (Ding et al. 2010). In the hematopoietic stem cells, ABCG2 may be involved in protection against hypoxic challenges (Krishnamurthy et al. 2004) and may permit enhanced stem cell survival in oxygen-poor environments by reducing the accumulation of toxic heme metabolites. These are the reasons why ABCG2 has been named as a "transporter for all seasons" (Sarkadi et al. 2004).

The Role of ABCG2 in Cancer Multidrug Resistance and ADME-Tox Parameters

In cancer cells the expression of ABCG2 results in chemotherapy resistance against a wide variety of anticancer agents that have to cross the cell membrane to reach their intracellular targets. In addition, it has been suggested that similarly to normal stem cells, the putative cancer stem cells (efficient cancer-initiating cells) preferentially express ABCG2, and this expression pattern may be an important factor in the inherent chemotherapy resistance of cancer stem cells. Similarly to that found in the case of the other key cancer multidrug transporter, ABCB1/MDR1/Pgp (see Chapter 'Genetic Polymorphisms of P-glycoprotein: Echoes of Silence'), a long list of chemotherapeutic agents testifies the huge variety of experimentally verified ABCG2 export substrates (e.g., mitoxantrone, camptothecin, methotrexate, daunorubicin, and doxorubicin—for details see (Brózik et al. 2011; Cusatis et al. 2006; Hegedus et al. 2009a).

A long sought-after solution to counteract cancer multidrug resistance has been the application of specific inhibitors of key ABC multidrug transporters. Several generations of high affinity, specific, or more general inhibitors have been developed and investigated in large clinical studies. However, these studies resulted in unexpected failures and drug developers became uninterested in this particular therapeutic intervention. The explanation of these failures is discussed in detail in (Szakacs et al. 2006), but in addition to several technical problems (e.g., improper diagnosis of transporter expression) the key limitation was an interference with the inherent physiological role of the ABC multidrug transporters—that is the protection of our body against chemical invaders.

ABCG2 is one of the key players in the chemical defense network of the human body. While its function is dispensable under the conditions of a protected environment (see below), full inhibition of this protein may lead to increased exposure

of pharmacological sanctuaries like the CNS during cancer chemotherapy. Inhibition of intestinal ABCG2 results in increased absorption of normally extruded toxins, and may strongly disturb the metabolic protection against untoward side-effects of xenobiotics. Thus, general inhibition of the ABCG2 protein may not be the optimal solution to improve the efficacy of cancer therapy even if high ABCG2-expressing cells are the targets.

An especially interesting area in this regard is the recently developed large number of targeted small molecule anticancer agents, including signal transduction or proteasome inhibitors. It has been documented for numerous such molecules that they are transported substrates of ABCG2 (or in some cases of ABCB1). These targeted agents have to reach their targets in the cell interior, and are usually hydrophobic and rapidly cross the plasma membrane. If they are ABCG2 substrates, in spite of their specifically targeted nature for affecting the mutated signaling or regulatory proteins and/or pathways, the cancer cells, and especially the cancer stem cells, are protected by the promiscuous transporter. Accordingly, overexpression (too much) of ABCG2 in cancer is an adverse prognostic factor for therapeutic response in numerous cancer types (see Dean et al. 2005; Robey et al. 2007; Chen et al. 2011). However, a potential hope in this regard is provided by the observations that several targeted anticancer agents, including, e.g., imatinib or nilotinib, are strong inhibitors of the ABCG2 protein at therapeutically relevant concentrations. Thus, the cancer-related cellular action of these molecules is not prevented by active extrusion and, in addition, they provide chemotherapy sensitivity for co-administered agents, otherwise exported by this ABC protein. Detailed in vitro and clinical studies may further explore this therapeutically important phenomenon.

ABCG2 Mutations, Polymorphisms, and Human Diseases Related to "Less" ABCG2

For a long time, ABCG2 expression has been implicated only in multidrug resistance, and, on the positive side, a protective mechanism against toxic xenobiotics. Polymorphic variants or nonsense mutations of ABCG2 were found to be associated with higher sensitivity and interindividual variability to various drug treatments (see Basseville et al. 2012). Numerous ABCG2 genetic variants have been studied in model cell lines. The most frequent/relevant polymorphic variant, Q141K ABCG2, has been shown to be associated with lower plasma membrane expression and/or reduced transport function (see Morisaki et al. 2005). Still, until recently, "too little" of ABCG2 has not been linked to specific human disease conditions.

In ABCG2 knock-out mice studies, when these animals were kept under controlled conditions, practically no phenotype was observed. Detailed experiments thereafter indicated that a diet containing the chlorophyll metabolite pheophorbide resulted in a UV-dependent skin damage as a result of the altered absorption and

metabolism (Vlaming et al. 2009). In the ABCG2 knock-out animals, the fetuses accumulate higher levels of cytotoxic drugs, and the concentration of cytotoxic agents and xenobiotics was increased in milk. Despite the increased sensitivity of Abcg2 KO mice to xenobiotics, the human disease-causing effect of less ABCG2 was thought to be not significant.

This paradigm was significantly changed by two key observations. The first was the recognition of a significant role of an ABCG2 polymorphism, the Q141K alteration in the development of gout by GWAS studies, and the demonstration of the role of the ABCG2 protein in uric acid metabolism (Woodward et al. 2009). Gout is a relatively human-specific condition, as in primates and humans, the end-product of the purine metabolism is uric acid, which can accumulate in the joints. ABCG2 is involved both in the renal and extrarenal transport of uric acid, thus counteracting systemic and local hyperuricemia (Matsuo et al. 2009, 2014; Ichida et al. 2012). The Q141K polymorphic variant of ABCG2, present with 5–30 % allele frequency in various human populations (Cervenak et al. 2006), has been shown already in 2005 to be less efficiently expressed in the plasma membrane of model cells (Morisaki et al. 2005; Mizuarai et al. 2004). Later, it was shown to undergo rapid degradation, and to possess less efficient transport properties (see Woodward et al. 2009). Together with both in vitro and in vivo data, by now it has been firmly established that human ABCG2 is an efficient uric acid transporter, and mutations or polymorphic variants, causing a lower level functional expression of this transporter, are causative in the development of gout (Dehghan et al. 2008).

The other unexpected discovery was the recognition of the ABCG2 protein as a blood group antigen. Two papers published within the same issue of Nature Genetics in 2012 (Saison et al. 2012; Zelinski et al. 2012) linked the rare blood group Jun- to the ABCG2 protein, showing that Jun- individuals have no ABCG2 expression in their red cell membranes. These individuals have mutations in their ABCG2 genes on both alleles, resulting in early termination of transcription. Although Jun- individuals have no apparent disease conditions, they may have anti-Jr(a) antibodies in their serum, which can cause transfusion reactions or hemolytic disease of the fetus or newborn (Saison et al. 2012). Corresponding to the higher incidence of ABCG2 nonsense mutations, the Jr- phenotype has a higher frequency in Asian populations.

Detection and Potential Correction of ABCG2 Protein Expression

As discussed above, polymorphic variants and mutations in ABCG2 may have important consequences regarding drug treatment, xenobiotic metabolism, the development of gout, or rare blood group related diseases. The related genetic diagnostics may help to devise chemotherapy protocols, combined medical treatments, or dietary precautions in a large number of patients (Basseville et al. 2012).

However, it has to be considered that ABCG2 expression is regulated by several signal transduction pathways, especially by nuclear receptors, and may be directly influenced by posttranslational modifications (Tóth et al. 2015). It has been documented for numerous membrane proteins, and clearly for ABCG2, that mRNA expression is not directly related to the level of protein expression, and especially not necessarily to proper plasma membrane localization (see Robey et al. 2007). Thus, in many cases the determination of ABCG2 protein expression levels in the relevant membrane of a specific tissue is warranted.

Diagnosis of "too much" ABCG2 in drug-resistant cancer relies on functional assays measuring fluorescent dye accumulation (see Hegedus et al. 2009b). "Too little" ABCG2, that is, reduction of ABCG2 expression and function cannot be directly deciphered from genetic analysis, as numerous minor polymorphisms, mutations, not easily detectable splicing problems, or other cellular processing alterations may variably alter membrane protein levels.

In order to approach this problem, we have recently developed a rapid, simple, and reliable, antibody-based flow cytometry assay for the quantitative determination of the ABCG2 protein in the human red blood cell (RBC) membranes (Kasza et al. 2012). We hypothesized that RBC expression may closely correlate with general tissue expression levels, as the potential genetic defects or processing problems should also affect ABCG2 expression during erythropoiesis, and the red cell membrane provides a long-term, relatively stable indicator for measuring this expression. Indeed, in healthy volunteers we have detected significant differences between the expression levels of the wild-type ABCG2 protein and the heterozygous Q141K polymorphic variant. In addition, we found several individuals with about 50 % reduction in RBC ABCG2 expression, and by sequencing the ABCG2 gene we found the related monoallelic nonsense mutations in these individuals. By now we have extended this observation to several other membrane proteins (see Várady et al. 2013; Koszarska et al. 2014) to suggest that the RBC membrane protein levels may be applied to detect genotype-dependent tissue expression patterns. Such a diagnostic approach may significantly facilitate appropriate therapeutic interventions.

As to the potential correction of reduced ABCG2 expression, studies related to the polymorphism causing both processing, functional or trafficking problems, as in the case of the Q141K variant, may help to devise rescue strategies. There have been numerous studies examining these cellular mechanisms (see Sarkadi et al. 2006). In our recent work (Sarankó et al. 2013) we have studied the stability and cellular processing of this variant and a related mutation (ΔF142 ABCG2), corresponding to the processing deficient ΔF508 mutation in the ABCC7 (CFTR) protein. Similarly to that seen in ABCC7, the Q141K variant had a mild processing defect, which could be rescued by low temperature and partially by a chemical chaperone (phenylbutyrate, PBA). Mutations and chemical interventions, resulting in a corrected folding, trafficking, and functional properties of ABCG2 may provide new hopes for rescue strategies.

The Enigmatic Localization and Function of ABCB6

Structure, Function, and Localization of ABCB6

ABCB6 is widely expressed in many tissues, especially in the heart, skeletal muscles (Mitsuhashi et al. 2000), and skin (Zhang et al. 2013). ABCB6 is a half-transporter of 842 amino acids, containing a unique N-terminal region followed by the ABC core consisting of a transmembrane domain and a cytoplasmic nucleotide-binding domain. ABCB6 forms homodimers (Krishnamurthy et al. 2006) and was shown to possess ATPase and transport activities after purification and functional reconstitution into liposomes (Chavan et al. 2013).

ABCB6 was first identified as the human ortholog of the yeast mitochondrial ABC transporter Atm1p (Mitsuhashi et al. 2000). In 2006, Krishnamurthy and colleagues published a paper showing that ABCB6 catalyzes the mitochondrial uptake of a heme synthesis intermediate, coproporphyrinogen III, thereby serving as an important regulator of cellular porphyrin biosynthesis (Krishnamurthy et al. 2006). Based on these findings ABCB6 is usually discussed in the context of mitochondrial ABC transporters, despite mounting evidence supporting its extramitochondrial localization and function. ABCB6 differs in several aspects from the three canonical (ABCB7, ABCB8, and ABCB10) inner membrane mitochondrial ABC transporters: it lacks a mitochondrial targeting sequence; humans and mice seem to tolerate its absence without any obvious phenotype; and its glycosylated form has been detected along the classical secretory pathway including the ER, the Golgi apparatus and the plasma membrane. ABCB7, ABCB8, and ABCB10 reside in the inner mitochondrial membrane, where they promote the export of various solutes from the mitochondrial matrix. Although the transported substrates remain to be identified, it is generally accepted that the function of the mitochondrial ABC transporters is linked to erythropoiesis and heme metabolism. ABCB7 and ABCB8 are believed to be involved in mitochondrial iron export (Allikmets et al. 1999; Ichikawa et al. 2012, 2014), and recent evidence suggests that ABCB10 plays an essential role in the protection against oxidative stress during erythropoiesis (Liesa et al. 2011, 2012).

As mentioned above, Krishnamurthy and colleagues assigned ABCB6 to the outer mitochondrial membrane with the nucleotide-binding domain facing the cytoplasm. This orientation implies an inward transport (i.e., mitochondrial import), which was consistent with experiments showing the uptake of ^{55}Fe-labeled hemin by mitochondria purified from ABCB6-overexpressing cells (Krishnamurthy et al. 2006). Although initial findings suggested that loss of one Abcb6 allele in embryonic stem (ES) cells impairs porphyrin synthesis (Krishnamurthy et al. 2006), mice derived from these stem cells were phenotypically normal (Ulrich et al. 2012). In 2012, the group of Arnaud Lionel at the National Institute of Blood Transfusion in Paris identified ABCB6 as the molecular basis of a rare blood group antigen called Langereis (Lan), and showed that Lan(−) individuals do not show any phenotype, suggesting that porphyrin import into the mitochondrial intermembrane

space may not be dependent on ABCB6 (Helias et al. 2012). Several other groups have identified ABCB6 in other extramitochondrial compartments, challenging the paradigm linking the expression and function of ABCB6 to mitochondria (Kiss et al. 2012). Based on these results it appears that ABCB6 is localized in the endolysosomal continuum including the plasma membrane (Helias et al. 2012; Paterson et al. 2007), the Golgi apparatus (Tsuchida et al. 2008), and organelles of the vesicular system (Bagshaw et al. 2005; Schroder et al. 2007; Della Valle et al. 2011; Jalil et al. 2008) such as secreted exosomes (Kiss et al. 2012). Regulation of the intracellular trafficking and the molecular details of targeting of the ABCB6 protein are not known. More work is needed to identify cellular and experimental conditions that result in the targeting of ABCB6 to mitochondria or the secretory pathway.

Expression and Function of ABCB6 in Pathological Conditions: Genotype–Phenotype Correlations

Elucidation of the intracellular targeting and trafficking of ABCB6 should provide hints to the physiological functions of ABCB6. Whereas lack of ABCB6 in Lan-negative individuals does not result in an overt phenotype, ABCB6 mutations have been associated with various pathological conditions such as ocular coloboma (Wang et al. 2012), dominant familial pseudohyperkalemia (Andolfo et al. 2013), and dyschromatosis universalis hereditaria (DUH) (Zhang et al. 2013). The genotype–phenotype correlations or the pathophysiological role of ABCB6 in these conditions are not known, as it has been difficult to correlate sequence variations to ABCB6 function or expression. This is partly due to the lack of our understanding of the physiological function and the structure–activity relationships within ABCB6, and to the complex regulation of synthesis, maturation, trafficking, and posttranslational modifications of membrane proteins.

Genotyping of cryopreserved blood showing weak or no reactivity with anti-Lan antibodies has identified 34 ABCB6 sequence variants in association with reduced ABCB6 expression. All the Lan-negative individuals genotyped so far have inherited two recessive null mutations. Heterozygous mutations result in reduced erythrocytic ABCB6 protein levels, indicating bi-allelic expression of ABCB6. It is not known how Lan mutations that do not result in gross sequence alterations result in lower or absent erythrocytic ABCB6 expression. Whereas the R192W mutation causes ER retention in model cells (Koszarska et al. 2014; Saison et al. 2013), other point mutations associated with reduced red blood cell expression did not influence the distinct endolysosomal expression pattern of ABCB6 (Koszarska et al. 2014). These results highlight the relevance of erythrocyte-specific trafficking events that may not be readily studied in cell lines that are routinely used to model protein expression and function. Erythroid cells undergo significant membrane remodeling during the late stages of differentiation. During this process proteins residing in

intracellular membrane compartments are either lost through secreted exosomes or are redistributed to the plasma membrane of mature erythrocytes.

Currently, there is no information available on the distribution of ABCB6 expression levels across the general population, healthy subpopulations, and disease-diagnosed individuals. Lan negativity is extremely rare, estimated to appear in approximately 0.005 % of the Caucasian population (Reid et al. 2014). Interestingly, ABCB6 expression levels in red blood cells display a large variation even in healthy individuals with an unexpectedly high frequency of low expressors, suggesting that genetic variations underlying low red blood cells ABCB6 expression are more common than inferred based on the frequency of Lan-negative blood type (Koszarska et al. 2014).

Ocular coloboma is a developmental defect of the eye linked to the abnormal or incomplete closure of the optic fissure. Mutations in two conserved residues of ABCB6 were identified by positional cloning and the targeted sequencing of the Abcb6 gene in sporadic cases of microphthalmia with coloboma (MAC) (Wang et al. 2012). ABCB6 is highly expressed in human retina and retinal pigment epithelial (RPE) cells. Confocal microscopy experiments found that the wild-type and mutant ABCB6 variants carrying the A57T and L811V mutations associated with coloboma are localized to the endoplasmic reticulum and Golgi apparatus of RPE cells. It remains to be shown how a 50 % reduction of the protein levels and/or function explains the developmental defects identified in heterozygous coloboma patients. Neither the wild-type nor the mutant proteins showed colocalization with mitochondria, suggesting that the coloboma mutations do not affect the protein's subcellular localization (Wang et al. 2012). The pathological relevance of coloboma mutations was demonstrated in zebra fish, where knockdown of ABCB6 produced a phenotype characteristic of coloboma. Importantly, the knockdown phenotype could be corrected with coinjection of wild-type ABCB6 suggesting that the phenotype observed in zebra fish is due to insufficient ABCB6 function (Wang et al. 2012). Despite the compelling results obtained in the zebrafish model, it is not clear how ABCB6 function is related to the formation and closure of the optic fissure. It is worth noting that a similar experiment examining the effect of DUH mutations (see below) did not reproduce any coloboma-related eye defects (Liu et al. 2014).

DUH is a pigmentary disorder characterized by hyperpigmented and hypopigmented macules distributed randomly over the body. The first DUH mutations were identified in families (L356P) and sporadic cases (S170G, G579E). Subsequently, the mutational spectrum of the ABCB6 gene was expanded through the analysis of further autosomal dominant (A453V, G555K) and sporadic DUH patients (459delC, 776delC, S322K) (Zhang et al. 2013; Liu et al. 2014; Cui et al. 2013). No difference was found in the expression of ABCB6 between patient and control samples. Electron microscopy revealed an abnormal pattern of mature melanosomes and immature melanosomes in the basal layer of hypo- and hyperpigmented skin obtained from a DUH patient (Cui et al. 2013). Expression of the tagged forms of the mutant and wild-type proteins in B16 melanoma cells revealed endosomal localization for the wild-type protein, and a distinct perinuclear localization corresponding to the Golgi apparatus in the case of the mutant ABCB6 variants (Zhang

et al. 2013). DUH is essentially a deficiency of melanin synthesis and/or melanosome sorting. Melanin synthesis takes place within melanosomes that are derived from the endoplasmic reticulum. Several melanogenic proteins are sorted in exosomes along melanosome maturing—the presence of ABCB6 in exosomes (Kiss et al. 2012) may be related to this process.

Functional gene mapping identified the segregation of missense ABCB6 mutations with familial pseudohyperkalemia (FP), which is a dominant red cell trait characterized by increased serum [K^+] in whole blood stored at or below room temperature (Andolfo et al. 2013). The reported FP mutations (R375Q and R375W) do not alter protein levels or localization. At present, it is unclear how ABCB6 mutations contribute to increased cation leak from red blood cells.

The absence of significant phenotypic alterations in ABCB6 loss-of-function models and in Lan-negative individuals demonstrates that hypomorphic and null mutations of ABCB6 expression are of only modest pathological importance under unstressed conditions (Andolfo et al. 2013). Yet heterozygous mutations at different positions in the *ABCB6* gene result in seemingly independent phenotypes: DUH mutations do not result in any phenotype associated with coloboma (and vice versa). Clearly, further studies are needed to explain how specific mutations associate with distinct phenotypes.

A Comprehensive Mutational Database, ABCMdb, for ABC Transporters

In order to design new drugs, strategies for rescuing the function or the proper localization of a specific mutant protein, knowledge on the effect of mutation, or the mutation at the same position in a homologous gene is an important prerequisite. During the last decade, numerous databases have been created to connect information on diseases, mutations, and their mechanisms. However, most of these databases exhibit serious shortcomings. First, most of the data collected focus on genetic alterations found in patients, while a plethora of mutations has also been experimentally generated. Since these mutations are well characterized in many aspects (effect on function, trafficking, etc.), they could serve as a comparative basis for targeting the disease-causing mutations observed in patients. Second, in many cases a specific mutation at a given position in the target protein has not been studied yet, but data on mutations at similar positions in homologous proteins from the same or different species are available. This information can also help investigations for rescuing mutant variants.

Recently we have generated a web application and a connected database (ABCMdb; http://abcmutations.hegelab.org) to facilitate structure/function studies of ABC proteins (Gyimesi et al. 2012). This database contains any mention of amino acid substitutions occurring in searchable full text files, as compared to the reference sequence, including natural and experimental mutations or

polymorphisms, found in genotypic or protein in vitro studies. The key features of this database include the searchable presentation of missense mutations at the protein level and the corresponding nucleotide numbers in the coding region, the sequences of homologous proteins with potential mutations in the related areas, and in many cases the available 3D structure information.

As a manual collection of the published mutations is highly resource expensive, we decided to automatically mine mutational data from the literature, in spite of the somewhat increased level of errors produced by such automatic approaches. In this regard it is important to note that a manual collection of data from publications and even the publications themselves are also tainted by human errors. During generation of the database containing ABCC6 variations (http://www.ncbi.nlm.nih.gov/lovd/home.php?select_db=ABCC6) approximately 40 % of the mutations mentioned in published papers turned out to be erroneous (e.g., typos either in the allele or locus—A. Váradi, personal communication). In an automatic collection, the pattern describing a protein mutation is relatively simple (a.a., position, another a.a.), while its correct identification is complex. It is generally assumed that the name of the protein harboring the mentioned mutation is close to the mutations described in the text (e.g., in the same sentence). Moreover, the a.a. at the given position can be confirmed by the reference sequence of the protein. Still we could experience the worst-case scenario, namely the protein mentioned in the same sentence with the mutation is not the target (mutated) protein but exhibits the same amino acid at the same position as the wild-type target protein. While the MutationFinder software (Caporaso et al. 2007) was used in the last years in different pipelines (Gyimesi et al. 2012; Vohra and Biggin 2013) to identify mutation patterns, the recent approach of tmVar (Wei et al. 2013) seems to exhibit a better performance. In addition, the analysis from any type of database must be performed very carefully because of human errors in the original publications.

Since a simple list of mutations identified for the target proteins is not user-friendly, implementation of a useful presentation layer is an important objective. To facilitate discoveries we provide three specific layers for researchers: (1) The sentences, in which the queried mutation was matched, are also listed together with the publication's PubMed ID that makes manual verification of the hit easier, if needed. (2) Deposited alignments of homologous sequences help both visualizing the a.a. position of interest in the target ABC protein in the context of mutations described in other ABC proteins, and searching for mutations in homologous and nearby positions. (3) Structural models for certain proteins and domains allow the users to investigate mutations in 3D, to decipher their possible effect at the atomic level.

In ABCMdb we elected to present only missense or nonsense point mutations in the coding region, as we have been focusing on studies at the protein level. There is an increasing demand to make additional information accessible, including insertions, deletions, and mutations in the non-coding regions, etc. Additional information on the effect of the mutation (e.g., disease associated, function disrupting, trafficking–effecting) would also effectively guide further research. However, automatic detection of nucleotide mutations is not really effective and decoding the

functional effects of mutations by employing language processing tools is currently not potent enough (Peterson et al. 2013).

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Two Liver Transporters, ABCB11 and ABCC6; Novel Therapeutic Approaches in the Related Disorders

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Abstract The bile salt export pump, ABCB11, selectively expressed in the hepatocytes, mediates the transport of monovalent bile salts into the bile. Mutations and polymorphisms in ABCB11 may lead to various forms of cholestatic liver diseases ranging from milder symptoms to lethal conditions. Similarly, inborn errors in ABCC6, another hepatic ABC transporter, may result in diverse conditions, which are characterized by soft tissue mineralization. In this chapter we review the recent knowledge on the structure, transport function, tissue distribution, transcriptional and posttranscriptional regulation, as well as the physiological and pathophysiological role of these medically important hepatic transporters. Substantial data on the pathomechanisms of the diseases associated with the dysfunction of these hepatic transporters have been accumulated in the recent years. This knowledge allows novel, mutation-specific therapeutic interventions opening a new perspective for the treatment of these diseases.

Keywords ABCB11/BSEP · ABCC6/MRP6 · Cellular localization · Trafficking · Functional correction

Introduction

Numerous ABC transporters are expressed in the liver playing pivotal role in various hepatic functions, such as bile secretion, detoxification, cholesterol metabolism, etc. ABC transporters residing in the canalicular (apical) membrane of hepatocytes, e.g., ABCB11/BSEP, ABCB4/MDR3, and ABCG5/G8, are responsible for the transport of the major bile constituents into the bile canaliculi, a luminal space formed by adjacent hepatocytes. The multidrug transporters,

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ABCB1/MDR1 (Pgp), ABCC2/MRP2 (cMOAT), and ABCG2, are also localized to the canalicular membrane, and play a role in detoxification by excretion of endo- and xenobiotics into the bile. A dissimilar set of ABC transporters is expressed in the basolateral membrane of hepatocytes, including ABCA1, ABCC1 (MRP1), and ABCC3-6 (MRP3-4). ABCA1 is a key component in the HDL biogenesis, whereas various MRPs may contribute to the elimination of toxic agents. The most enigmatic of these is ABCC6, whose function is not fully understood.

In this chapter we focus on two hepatic transporters, the canalicular ABCB11, the dysfunction of which leads to various cholestatic liver diseases; and the basolateral ABCC6, impaired function of which results in disorders characterized by increased soft tissue mineralization. A large number of mutant and polymorphic variants are known for both ABCB11 and ABCC6. Many of them have an etiological role in diseased conditions, whereas several others are only susceptibility factors for the development of disorders associated with the genetic variants of these transporters. Better understanding of the function of these medically important transporters, and of the mechanism how mutations/polymorphisms in these transporters lead to the disorders may assist to develop novel therapeutic approaches to cure these diseases.

Physiological and Pathophysiological Role of ABCB11 (BSEP)

Structure, Functional Characteristics, Tissue Distribution, and Localization of ABCB11

The majority of bile constituents are continuously cycled from the liver through the intestine to the bloodstream. Numerous ABC transporters significantly contribute to the enterohepatic circulation at each stage—including hepatic secretion, intestinal absorption, and even, though indirectly, at the serosal uptake in the liver. The rate-limiting step of bile formation is the secretion of bile acids (or bile salts) through the apical (canalicular) membrane of hepatocytes (Stieger and Beuers 2010), driven predominantly by a liver-specific ABC transporter ABCB11, also termed bile salt export pump (BSEP).

ABCB11 is a member of the B subfamily of ABC transporter proteins, closely related to ABCB1 (MDR1, P-glycoprotein), hence, it was originally named as sister of P-glycoprotein (Spgp), when cloned from a pig liver cDNA library in 1995 (Childs et al. 1995). ABCB11 possesses canonical membrane topology with two NBDs and two TMDs, containing a relatively large, glycosylated extracellular loop between transmembrane helices 1 and 2, resembling ABCB1 (MDR1).

ABCB11 is an active transporter driven by the energy of ATP binding and hydrolysis, however, contrary to ABCB1 (MDR1) or ABCG2, ABCB11 has a narrow substrate specificity. The primary substrates of ABCB11 are bile salts

including taurine- and glycin-conjugates, e.g., taurocholate, as well as unconjugated bile salts such as chenodeoxycholate or deoxycholate (Byrne et al. 2002; Noe et al. 2002). This transport function of ABCB11 seems highly conserved, however, minor differences between human and non-human orthologs have been reported (Hayashi et al. 2005; van Beusekom et al. 2013). Although the transport of monovalent bile salts is the predominant function of ABCB11, non-bile acid compounds, such as vinblastine, calcein-AM, pravastatin, and fexofenadine, have also been identified as substrates (Lecureur et al. 2000; Hirano et al. 2005; Matsushima et al. 2008).

The ABCB11 protein is selectively expressed in the liver (Childs et al. 1995), although its transcript was also found in non-hepatic tissues including testis in humans (Langmann et al. 2003); brain cortex, small and large intestine in rats (Torok et al. 1999); as well as the kidney in sea lamprey (Cai et al. 2012). Comprehensive immunohistochemistry analysis of human tissues verified the predominant hepatic expression of ABCB11, but low level of protein expression was also found in the testis and the adrenal gland (Uhlen et al. 2010). The function of ABCB11 in the latter tissues remains unclear. In the liver, ABCB11 is expressed in the canalicular membrane and subcanalicular vesicles of hepatocytes (Gerloff et al. 1998; Dombrowski et al. 2006). Interestingly, the majority of ABCB11 resides in the intracellular pool under basal conditions (Kipp et al. 2001), but can be mobilized to the canalicular plasma membrane upon stimulation (see below). Trafficking studies also revealed that ABCB11 continuously cycles between a rab11a-positive endosomal pool and the canalicular membrane compartment (Wakabayashi et al. 2004). Contrary to other canalicular membrane proteins, such as the transferrin receptor, following biosynthesis ABCB11 is directly targeted from the Golgi compartment to the canalicular surface (Kipp and Arias 2000).

Regulation of ABCB11 Expression and Function

As other components of the hepatocellular transport system, ABCB11 is tightly regulated at both transcriptional and posttranscriptional levels. Whereas the former accounts for long-term changes in transporter activity, the posttranscriptional control can act rapidly. Intracellular accumulation of bile salts can trigger both regulatory mechanisms. The major transcriptional stimulator of ABCB11 is the bile salt-sensing nuclear receptor, FXR, which acts through its heterodimerization with RXRα, and binding to the promoter of ABCB11 (Plass et al. 2002). Additional transcriptional regulation of ABCB11 is accomplished by several nuclear receptor co-activating proteins, such as ASCOM, CARM1, and SRC2, modifying the FXR-mediated response. Other mechanisms independent of FXR, including regulation via LRH1, NRF2, and PXR, have also been reported (for review see: Baghdasaryan et al. 2014).

Cell surface expression of the ABCB11 can rapidly be elevated when higher bile secretory capacity is required. This quick response takes place through the

redistribution of ABCB11 from the intracellular reservoir to the canalicular membrane compartment. Taurocholate and cAMP have been shown to additively increase the canalicular expression of ABCB11 (Kipp et al. 2001), suggesting two independent mechanisms. A recent trafficking study revealed the co-existence of an LKB1/AMPK- and a PKA-dependent pathway controlling the canalicular delivery of ABCB11 (Homolya et al. 2014). Taurocholate was found to stimulate ABCB11 trafficking solely through the LKB1-regulated pathway. Studies on isolated rat liver also established a regulatory role for PI3K in the taurocholate-dependent membrane insertion of ABCB11 (Misra et al. 1998), whereas PI3K was not involved in the cAMP-induced redistribution of the transporter (Misra et al. 2003). In addition, another bile acid, tauroursodeoxycholate, was shown to stimulate ABCB11 canalicular trafficking through a mechanism, which involves p38 MAP kinase and Ca^{2+}-independent PKC isoforms (Kubitz et al. 2004). The role of myosin II regulatory light chain in the apical delivery of newly synthesized ABCB11 has also been demonstrated (Chan et al. 2005). Several additional regulatory proteins, including HAX-1, cortactin, EPS15, AP-2, and type II IP3-receptor, have been shown to contribute to the anchoring and internalization of ABCB11, influencing its half-life at the apical cell surface (for more details see Soroka and Boyer 2013).

In addition to this complex, fine control of trafficking to the cell surface, the activity of the transporter can directly be modulated by posttranslational modifications or through its lipid environment, as also established for ABCB1 (MDR1). Direct phosphorylation of ABCB11 was demonstrated in Sf9 insect cells (Noe et al. 2001), although its regulatory role is still elusive. Interestingly, estradiol-17β-glucuronide and several progesterone metabolites are able to inhibit the ABCB11-mediated bile salt transport from the extracellular side (trans-inhibition) (Stieger et al. 2000; Vallejo et al. 2006). Furthermore, the activity of ABCB11 was found to be dependent on the cholesterol content of membrane microdomains (Paulusma et al. 2009). A recent study demonstrated that cholesterol elevates V_{max} without affecting K_m of the ABCB11-mediated taurocholate transport (Guyot et al. 2014). However, the actual physiological significance of these posttranslational regulatory mechanisms, especially in humans, remains to be elucidated.

ABCB11 Mutations and Their Role in Human Hereditary Diseases

More than 100 variants of ABCB11 have been identified thus far (see in Gyimesi et al. 2012). Among those missense, nonsense, and splice-site mutations, deletions, and insertions, as well as synonymous and nonsynonymous single nucleotide polymorphism (SNPs) are described (Strautnieks et al. 2008; Lang et al. 2006; Ho et al. 2009). These mutations and SNPs are spread out throughout the entire protein, but the most affected regions are the NBDs and the cytoplasmic loops. Loss-of-function mutations in ABCB11 lead to various forms of cholestatic liver

disease ranging from milder through intermittent symptoms to lethal progressive conditions, collectively named as "BSEP deficiency syndrome" (Pauli-Magnus et al. 2005). Common characteristics of these disorders are the reduction or complete lack of functional ABCB11 at the canalicular membrane (Strautnieks et al. 2008), which results in impaired bile formation and bile flow, intrahepatic accumulation of bile salts, consequently high bile salt and bilirubin plasma levels.

The most severe form of cholestatic liver diseases associated with ABCB11 mutations is known as progressive familial intrahepatic cholestasis type II (PFIC2), which is characterized by an early onset of cholestasis, and rapid progression to end-stage liver disease before adulthood. Infants with PFIC2 have recurrent or permanent jaundice associated with hepatomegaly, suffer from severe pruritus, fail to thrive, develop cirrhosis, and carry high risk for hepatobiliary malignancies (Jacquemin 1999; Knisely et al. 2006). Benign recurrent intrahepatic cholestasis type II (BRIC2) represents a milder form of BSEP deficiency syndrome, typically manifests in adulthood, and brings about repeated episodes of cholestasis, resulting in severe pruritus but mostly without marked liver damage. Nevertheless, certain BRIC cases evolve to a more aggressive form of cholestatic liver disease (van Ooteghem et al. 2002), and patients with intermediate phenotype between BRIC2 and PFIC2 have also been reported (Lam et al. 2006; Stindt et al. 2013). The clinical manifestation of PFIC2 and BRIC2 closely correlates with the cell surface expression of ABCB11 (Lam et al. 2007).

Disease-associated mutations in ABCB11 cause impaired bile salt secretion through various mechanisms including defective transport capability, impaired trafficking, protein misfolding, or increased proteasomal degradation (Lam et al. 2007; Wang et al. 2002; Noe et al. 2005; Kagawa et al. 2008; Plass et al. 2004). The two most frequent ABCB11 mutations, E297G and D482G, found in nearly 60 % of the European families affected by PFIC2 (Strautnieks et al. 2008), have been reported to result in defective canalicular trafficking (Hayashi et al. 2005), while other studies suggested that trafficking of these mutants is unaffected (Lam et al. 2007; Kagawa et al. 2008). A comprehensive study demonstrated normal trafficking for D482G but not for E297G (Wang et al. 2002). The controversy of these studies might be attributed to the difference in the applied expression systems possessing diverse trafficking machinery. Whether the transport activity of these mutants is also reduced or preserved, is even more controversial (Lam et al. 2007; Wang et al. 2002; Kagawa et al. 2008; Plass et al. 2004; Hayashi et al. 2005). Other common mutations in ABCB11, such as G982R, R1153C, and R1268Q, result in both trafficking and transport defects (Ho et al. 2009; Wang et al. 2002), whereas the G238V mutant form undergoes rapid proteosomal degradation, despite its normal transport function (Wang et al. 2002). In contrast, the R1057X mutant shows normal cell surface expression, but a complete loss of taurocholate transport (Kagawa et al. 2008; Plass et al. 2004). It has been proposed that reduced cell surface expression of most PFIC2 and BRIC2 mutants is due to the defect in protein stability, rather than in trafficking (Kagawa et al. 2008; Plass et al. 2004). Aberrant mRNA splicing has also been demonstrated for several ABCB11 mutations and SNPs, resulting in reduced mRNA and protein levels (Byrne et al. 2009).

Genomic Variations of ABCB11 and Acquired Cholestatic Liver Diseases

In addition to these hereditary cholestatic liver diseases, there are several forms of acquired diseases associated with impaired bile salt transport. The intrahepatic cholestasis of pregnancy (ICP) is a transient cholestasis, resulting in elevated serum bile acid, pruritus with variable severity, and increased risk for preterm delivery, meconium, and fetal distress (Pusl and Beuers 2007). ICP typically begins in the third trimester of pregnancy, when the serum level of estrogens is high. This is in line with the trans-inhibition of ABCB11 function by estrogen and progesterone metabolites; however, recent studies elucidated the importance of the transcriptional regulation of ABCB11 in the etiology of ICP by demonstrating inverse correlation between serum 17β-estradiol and ABCB11 levels (Song et al. 2014), as well as FXR-dependent repression of ABCB11 expression by 17β-estradiol and epiallopregnanolone sulfate (Song et al. 2014).

Several ABCB11 mutations and polymorphisms with ethnic-dependent allele frequencies have been identified as susceptibility factor for the development of ICP (Ho et al. 2009; Dixon et al. 2009; Eloranta et al. 2003; Keitel et al. 2006; Meier et al. 2008; Pauli-Magnus et al. 2010). Among those, the most common polymorphic variant, V444A, is concomitant with reduced mRNA and protein levels (Byrne et al. 2009), but the transport function of the protein is preserved (Ho et al. 2009). In contrast, several other variants, such as I206V, Q558H, N591S, and E1186K, result in significantly reduced transport activity, while the trafficking of E1186K variant is also impaired (Ho et al. 2009). Despite the unambiguous association between these ABCB11 polymorphisms and ICP, their role in etiology remains to be established.

The cholestatic form of drug-induced liver injury (DILI) can often be attributed to the inhibition of ABCB11 function (Dawson et al. 2011; Morgan et al. 2013). Similar to other forms of intrahepatic cholestasis, ABCB11 inhibition by drugs leads to accumulation of bile salts causing liver damage. Several agents, including high-profile drugs, as exemplified by rifampicin, troglitazone, nefazodone, glibenclamide, cyclosporin, and bosentan, have been shown to block ABCB11. Estrogen and progesterone metabolites secreted by ABCC2 (MRP2) to the bile canaliculi can also trans-inhibit ABCB11 (Stieger et al. 2000). Moreover, bosentan has a dual action: in addition to ABCB11 inhibition, it stimulates ABCC2 (MRP2) function, causing a bile salt-independent increase in bile flow (Mano et al. 2007). The prediction of toxicity of these drugs is crucial, but often erratic. A recent study demonstrates the applicability of a multiscale mechanistic model, DILIsym, estimating the frequency and severity of bile acid-mediated DILI, caused by ABCB11 inhibitors (Woodhead et al. 2014). The relatively low frequency of adverse drug reactions suggests the involvement of genetic susceptibility factors. Several rare mutations and the common V444A polymorphism have been identified as risk factors for drug-induced cholestasis (Pauli-Magnus et al. 2010; Lang et al. 2007). The pathogenic relevance of the V444A variant is further underlined by the

observation that all the four patients examined with contraceptive-induced cholestasis were found to be homozygous carriers of this polymorphism (Meier et al. 2008).

Other diseases associated with ABCB11 mutations and polymorphisms include primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), primary intrahepatic stones, and gallstone disease (Pauli-Magnus et al. 2004; Chen et al. 2014; Pan et al. 2014; Hirschfield et al. 2013). Association of certain ABCB11 variants with neonatal cholestasis has also been reported, although the most common SNPs, such as V444A, were not more frequent in the studied cohort (Liu et al. 2013). PFIC-associated mutations in ABCB11 are concomitant with susceptibility to develop pediatric hepatocellular carcinoma (HCC) and cholangiocarcinoma (Knisely et al. 2006; Davit-Spraul et al. 2010; Scheimann et al. 2007). The suggested mechanism of these malignancies is that dysfunction of ABCB11 results in intrahepatic accumulation of bile acids and chronic inflammation, which in turn cause extensive genomic modifications, promoting cancer (Iannelli et al. 2014). However, a recent study identified somatic mutations in a pediatric hepatocellular carcinoma patient carrying compound heterozygous ABCB11 mutations, with preserved protein expression, and normal serum bile salt level (Vilarinho et al. 2014). Despite the increasing number of genomic analyses demonstrating association between these conditions and ABCB11 mutations/SNPs, the functional relevance and the molecular mechanisms leading to acquired cholestatic diseases remain unclear.

Therapeutic Aspects of ABCB11-Related Cholestatic Liver Diseases

Since the manifestations of various disorders caused by ABCB11 defects range from mild symptoms to end-stage liver failure, the therapies for these conditions are also diverse. Drug treatments of PFIC2 are of limited effectiveness, thus, surgical interventions represent the major line of therapy. These include partial biliary diversion (external or internal), ileal bypass, and when the progression of the disease calls for it, even liver transplantation. Hepatocyte transplantation in PFIC2 is contraindicated by the potential risk of leaving premalignant cells in place. Even liver transplantation can occasionally fail, due to the emergence of anti-ABCB11 antibodies resulting in the recurrence of the BSEP deficiency phenotype (Keitel et al. 2009; Jara et al. 2009; Maggiore et al. 2010). Severe ABCB11 mutations (nonsense and splice-site mutations) are predominant in these posttransplant relapse cases (Kubitz et al. 2015). Interestingly, the outcome of partial biliary external diversion is also associated with the type of ABCB11 mutations; patients bearing E297G or D482G missense mutation exhibited better prognosis after this surgical intervention (Davit-Spraul et al. 2010; Pawlikowska et al. 2010).

Biliary diversion or ileal bypass reduces the accumulation of toxic bile acids by partial removal of the bile from the enterohepatic circulation. Cholestyramine, an anion-exchange resin used in symptomatic treatment for cholestatic liver disorders, acts similarly, hindering reabsorption and stimulating fecal excretion of bile acids. Rifampicin, an antibiotic and human PXR agonist, is also used to reduce pruritus in PFIC and benign recurrent intrahepatic cholestasis (BIRC). It can effectively abort the cholestatic episodes in numerous BRIC patients (van Ooteghem et al. 2002; Cancado et al. 1998; Folvik et al. 2012) likely by inducing CYP3A4, UGT1A1, and ABCC2 (MRP2), which results in increased urinary excretion of bile salt metabolites (Marschall et al. 2005). Ursodeoxycholic acid (UDCA) can also relieve symptoms and significantly improve clinical parameters in various forms of cholestatic liver diseases. However, the outcome of UDCA treatment in PFIC2 and BRIC2 is erratic, ranging from no response to clear improvement, nevertheless, UDCA is considered in the initial therapeutic management of PFIC to prevent liver damage (reviewed in Jacquemin 1999). Large cohort studies demonstrated that UDCA treatment significantly reduced pruritus in ICP patients, albeit the degree of its beneficiary effect remains controversial (Bacq et al. 2012; Chappell et al. 2012).

Numerous mechanisms have implicated to explain the mode of action of UDCA (reviewed in Festi et al. 2007). This natural, tertiary bile acid is less toxic than its more hydrophobic counterparts, thus, orally administered UDCA can partially replace the cytotoxic bile salts in the endogenous bile acid pool. Other suggested mechanisms include stabilization of hepatocyte membrane, stimulation of bile flow through cholehepatic shunting, inhibition of hepatocyte apoptosis, and immunomodulation. In addition, UDCA has been shown to induce ABCB11 and ABCC2 expression at the transcriptional level (Marschall et al. 2005; Fickert et al. 2001), and to increase the apical presence of ABCB11 by reducing its internalization (Kagawa et al. 2014). In contrast, taurine-conjugate of UDCA elevates cell surface expression of ABCB11 and ABCC2 by stimulating their canalicular delivery (Dombrowski et al. 2006; Kubitz et al. 2004).

Novel therapeutic approaches to treat ABCB11-related cholestatic liver diseases comprise the transcriptional activation by nuclear receptor agonists (reviewed in Baghdasaryan et al. 2014), and the assisted cell surface delivery of ABCB11 mutants using molecular chaperones (reviewed in Soroka and Boyer 2013). Activation of FXR by natural, synthetic, or semi-synthetic high-affinity ligands results in a simultaneous and coordinated response in expression of transporters and enzymes involved in the bile salt metabolism. Other potential transcriptional targets for the therapy of cholestatic liver disorders include CAR, PXR, PPARα, PPARγ, as well as the glucocorticoid and vitamin D receptors (see Baghdasaryan et al. 2014). Since certain ABCB11 mutations lead to misfolded protein, which are retained in the ER (Wang et al. 2002), it is feasible to employ correctors similar to those divulged in connection with CFTR ΔF508 (reviewed in Rudashevskaya et al. 2014) to rescue folding-defective ABCB11 mutants. In vitro studies demonstrated that pharmacological chaperones such as sodium butyrate, or 4-phenylbutyrate (4-PBA) can increase the cell surface expression of the common D482G and E297G mutant forms of ABCB11 (Lam et al. 2007; Hayashi and Sugiyama 2007). Even

bile salts have been shown to act as correctors of the E297G mutant (Misawa et al. 2012). Recently, two clinical studies demonstrated the beneficiary effect of 4-PBA (Gonzales et al. 2012; Naoi et al. 2014). The clinical and biochemical parameters have been substantially improved in PFIC2 patients bearing T1210P or R1231Q mutation in response to high-dose 4-PBA treatment. Increased canalicular expression of the mutant ABCB11 has also been demonstrated in these cases. In conclusion, development of high-efficacy correctors and mutation-specific therapeutic interventions represent new perspectives for the treatment of cholestatic liver disorders.

ABCC6 Mutations and Potential Conformational Therapy in Related Disorders

Structure, Functional Characteristics, Tissue Distribution, and Localization of ABCC6

ABCC6, a member of the MRP/ABCC-family, is expressed at a high level in the liver and localized in the basolateral compartment of the hepatocyte plasma membrane (Pomozi et al. 2013). This protein consists of 1503 amino acids and is embedded into the plasma membrane with 17 transmembrane helices. ABCC6 shows ATP-dependent organic anion transport activity (Ilias et al. 2002; Belinsky et al. 2002), though its physiological substrate is currently not known. Mutations in the *ABCC6* gene cause pseudoxanthoma elasticum (PXE), a multiorgan mineralization disorder, while it is the disease gene in some cases of generalized arterial calcification of infancy (GACI).

Regulation of ABCC6 Expression

Since expression of ABCC6 is the highest in the liver, the regulation of transcription of the gene has been investigated in vitro mostly in hepatocyte (HepG2) cell cultures. Both the signal transduction pathway of regulation as well as DNA structures harboring regulatory sites have been revealed (Aranyi et al. 2005; de Boussac et al. 2010; Ratajewski et al. 2012). The master tissue-specific regulator of ABCC6 expression is hepatocyte nuclear factor 4α (HNF4α), interacting with an evolutionarily highly conserved degenerate DNA site between −209/−145. This site overlaps with a DNA methylation-dependent activatory region (−332/+72) identified earlier (Aranyi et al. 2005). The important role of CCAAT/enhancer-binding protein beta (C/EBPβ) by binding to the primate-specific sequence in the first intron has also been shown (Ratajewski et al. 2012).

Growth factors, like HGF, inhibit the expression of ABCC6 by attenuating HNF4α binding to the promoter via a pathway involving the activation of the ERK1/2 cascade. In the first intron, the activator sequence binds C/EBPβ together with another, so far unidentified protein. These proteins interact with a complex formed by HNF4α binding to the promoter, activating the transcription of the gene. Oxidative stress has a negative impact on the expression of the gene (de Boussac et al. 2010)—interestingly, down-regulation of the mouse gene was observed in a beta-thalassemia mouse model (Martin et al. 2011), that may explain the development of secondary PXE in human beta-thalassemia patients, described earlier (Aessopos et al. 1992).

Diseases Associated with Soft-Tissue Mineralization

Ectopic (soft-tissue) mineralization can be developed due to aging and environmental factors, such as smoking or high fat diet. Mendelian disorders with soft-tissue calcification may provide important clues regarding the function of proteins involved in this complex pathological condition. According to the current view, soft tissue mineralization is controlled by a metabolic pathway stabilizing the plasma phosphate (Pi)/pyrophosphate (PPi) ratio. Pi facilitates, while PPi inhibits calcium hydroxyapatite crystal nucleation and growth. Calcification may also be affected by the local environment of the tissue. The first metabolite of this network is ATP, released from liver cells, and this release is facilitated by the plasma membrane transporter ABCC6 (Jansen et al. 2013, 2014), however, a direct transport of ATP by ABCC6 has not been demonstrated. ATP is immediately hydrolyzed in the liver vasculature to AMP and PPi by an ectonuclease, ENPP1 (Jansen et al. 2014). Mutations in the *ENPP1* gene result in generalized arterial calcification in infancy (GACI), an early onset inborn disorder with massive calcification of the vascular system (Rutsch et al. 2003). It is noteworthy, that in a small cohort of GACI patients no mutations were found in the *ENPP1* gene, rather *ABCC6* mutations are the genetic background of the disorder in these families (Nitschke et al. 2012). The expression spectrum of ENPP1 is wider than that of ABCC6, but ENPP1 expression was also found high in the liver. Since approx. 60 % of the plasma PPi is provided by the liver, PPi levels are low in both PXE and GACI patients (Jansen et al. 2014; Rutsch et al. 2003). The associated disorders are clearly different in their clinical impact: PXE (frequency is ∼1:50,000) is a late onset inherited disease with manifestation in the skin, in the eyes, and in the arteries, while GACI (very rare) is an often fatal condition with severe prenatal aortic calcification. Clearly, GACI and PXE represent the two extreme ends of a clinical spectrum of calcification disorders. PXE may serve as a genetic model to unravel the pathomechanisms of common, "acquired", age-associated degenerative diseases, like macular degeneration and vascular calcification.

In tissue mineralization a subsequent step is the hydrolysis of AMP to Pi and adenosine, catalyzed by a membrane-bound ecto-5′-nucleotidase (NT5E/CD73) at the site of soft tissue calcification. Adenosine down-regulates the expression of tissue non-specific alkaline phosphatase (TNAP), which cleaves PPi to 2Pi molecules. Thus, the unregulated, high activity of TNAP (in the absence of adenosine) results in an elevated Pi/PPi ratio, and ultimately increased calcification. The human condition associated with mutations in the *NT5E/CD73* gene is calcification of joints and arteries (CALJA) also called arterial calcification due to deficiency of CD73 (ACDC) (Hilaire et al. 2011).

ABCC6 Mutations Related to Transport Activity or Protein Trafficking

Clinical conditions (i.e., genetic diseases) and genetic risk factors are often due to a single missense mutation in a given gene. Such mutations may result in inactive, but stable protein, or in a protein variant with (at least partial) preserved activity, but with altered conformation (or the combination of thereof). In the latter case, due to lower stability, the half-life of such a polypeptide is short, and usually it cannot reach the physiological location within or outside the cell where its physiological activity is normally executed. Indeed, ABCC6 mutations causing PXE and GACI are mostly missense, frequently resulting in incorrect cellular localization into the plasma membrane, but not harming its transport activity in the case of ABCC6 (Le Saux et al. 2011; Pomozi et al. 2014). In principle, such mutants are considered as candidates for pharmacological "folding-correction" in order to guide them to their normal physiological location. Simple calculation shows that at least 75 % of PXE patients are with at least one *ABCC6* allele with missense mutation.

Potential Methods for the Correction of ABCC6 Function

Chemical chaperones have the notable advantage of promoting folding with no direct interaction with the proteins, thus not interfering with their function. As they probably act on the expression of ER-resident chaperone proteins—which assist the folding of the polypeptides, thus facilitating ER export and quality control—they may be toxic by perturbing this delicate and important system. However, if we restrict the search for chemical chaperons to a library of approved drugs, candidate compounds with known toxicity and other basic pharmacological parameters can be identified.

Several studies have also shown that 4-phenylbutyrate (4-PBA) can partially rescue the intracellular trafficking of the frequent DeltaF508 variant of

ABCC7/CFTR in vitro (Rubenstein et al. 1997). Of particular interest is that 4-PBA is approved by U.S. Food and Drug Administration for clinical use in urea cycle disorders and thalassemia (Maestri et al. 1991; Dover et al. 1992). Many membrane proteins with disease-causing mutations have also been successfully subjected to vitro to 4-PBA treatment, resulting in marked improvement of their folding/trafficking and recently, case reports of successful clinical utilization of 4-PBA to correct the mutation effects on the bile salt export pump (ABCB11/BSEP) in a patient suffering from progressive familial intrahepatic cholestasis type 2 were published (Gonzales et al. 2012; Naoi et al. 2014). In line with these results, recently we have published that disease-causing missense ABCC6 mutants accumulating intracellularly can be redirected into the plasma membrane in vivo in mouse liver using 4-phenylbutyrate (4-PBA) (Le Saux et al. 2011; Pomozi et al. 2013).

To investigate whether the correction of the cellular localization by 4-PBA could also restore the physiological (i.e., anti-calcification) function of ABCC6, we monitored the previously described dystrophic cardiac calcification (DCC) phenotype (Aherrahrou et al. 2007) as a rapid and easily quantifiable marker of the physiological function of ABCC6. We and others have established that DCC is an ABCC6-dependent phenotype (Aherrahrou et al. 2007; Brampton et al. 2014) occurring in Abcc6-deficient mice, but not in animals with normal hepatic ABCC6 expression. Furthermore, we found that transient expression of the human ABCC6 in the liver of Abcc6$^{-/-}$ mice dramatically reduced (by 62 %) the level of DCC (Brampton et al. 2014). Our preliminary results indicate that while the expression of three disease-associated mutants did not affect the development of DCC phenotype in mice, the concomitant administration of 4-PBA resulted not only in the reorientation of the protein into the plasma membrane, but also in the reduction of cardiac calcification to the same level achieved with expression of the WT ABCC6.

Manifestations of PXE symptoms impact dramatically the patients' quality of life. GACI, however, radically reduces life expectancy of the affected individuals and there is no effective therapy available for these two inherited calcification disorders. The re-routing of ABCC6 missense mutants into the plasma membrane of hepatocytes, and the (potentially) resulting rescue of ectopic calcification via 4-PBA treatment in mice demonstrate that an allele-specific therapy for human PXE and GACI patients is feasible. Drug repurposing currently receives a great deal of attention as an obvious way to reduce cost and shorten time for developing new therapies for rare diseases. To utilize this approach in "conformational therapy" has a blooming future. We have demonstrated the feasibility of the repurposing the use of 4-PBA for allele-specific conformational therapy to correct the pathophysiological calcification caused by human missense ABCC6 mutations associated with both PXE and GACI (Figs. 1 and 2).

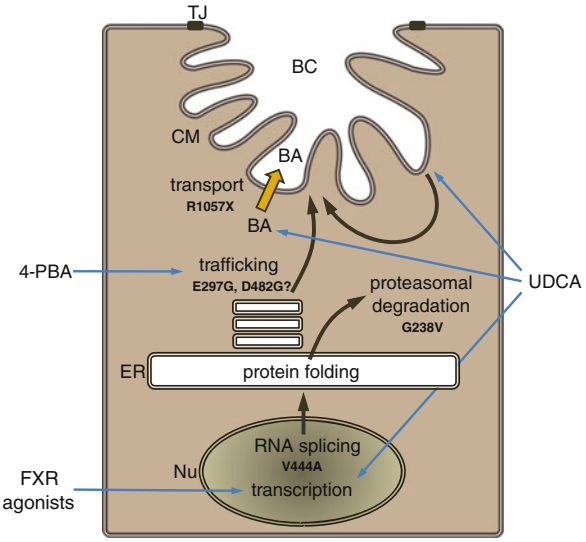

Fig. 1 Different mechanisms of how various mutations and polymorphisms in ABCB11 can affect the normal life cycle and function of the transporter (*dark arrows*). Different cellular targets in medical treatments of "BSEP-deficiency" (*blue arrows*). *Nu* nucleus, *ER* endoplasmic reticulum, *CM* canalicular membrane, *BC* bile canaliculus, *TJ* tight junction, *BA* bile acid. For more details see text

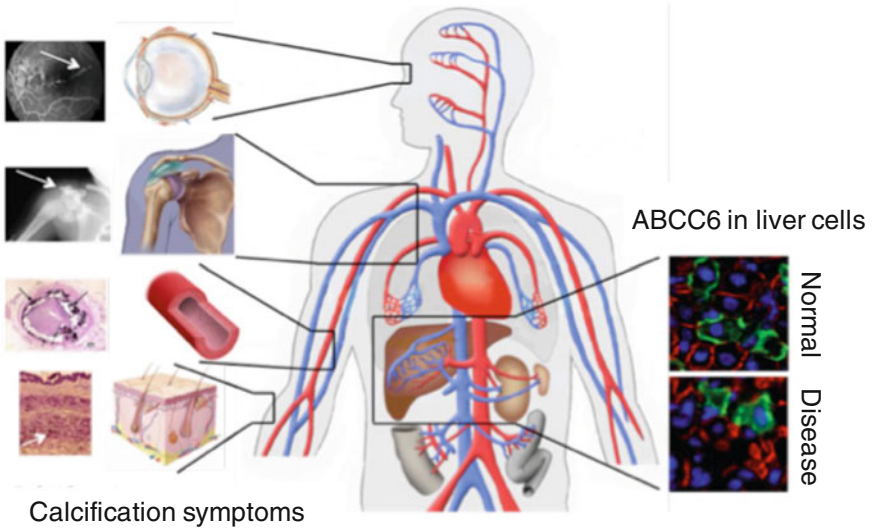

Fig. 2 Tissues and organs affected by mineralization in disorders PXE and GACI due to mutations in ABCC6

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