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Membrane Transporters: Structure, Function and Targets for Drug Design

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Abstract Current therapeutic drugs act on four main types of molecular targets: enzymes, receptors, ion channels and transporters, among which a major part (60–70%) are membrane proteins. This review discusses the molecular structures and potential impact of membrane transporter proteins on new drug discovery. The three-dimensional (3D) molecular structure of a protein contains information about the active site and possible ligand binding, and about evolutionary relationships within the protein family. Transporters have a recognition site for a particular substrate, which may be used as a target for drugs inhibiting the transporter or acting as a false substrate. Three groups of transporters have particular interest as drug targets: the major facilitator superfamily, which includes almost 4000 different proteins transporting sugars, polyols, drugs, neurotransmitters, metabolites, amino acids, peptides, organic and inorganic anions and many other substrates; the ATP-binding cassette superfamily, which plays an important role in multidrug resistance in cancer chemotherapy; and the neurotransmitter:sodium symporter family, which includes the molecular targets for some of the most widely used psychotropic drugs. Recent technical advances have increased the number of known 3D structures of membrane transporters, and demonstrated that they form a divergent group of proteins with large conformational flexibility which facilitates transport of the substrate.

Keywords Three-dimensional structure · Drug discovery · Drug targets · Membrane proteins · Transporters

Abbreviations

ABC	ATP-binding cassette
ATP	Adenosine triphosphate
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
2D	Two dimensional
3D	Three dimensional
DAACS	Dicarboxylate/amino acid:cation symporter
DAT	Dopamine transporter
DHA1	Drug:H^+ antiporter-1
DMT	Drug/metabolite transporter
DNA	Deoxyribonucleic acid
EAAT	Excitatory amino acid transporter
E-MeP	European Membrane Protein Consortium
EU	European Union
GABA	Gamma-aminobutyric acid
GAT	GABA transporter
GLUT	Glucose transporter
HAE	Hydrophobe/amphiphile efflux
HIV	Human immunodeficiency virus
5-HT	5-Hydroxytryptamine (serotonin)
MFS	Major facilitator superfamily
MS	Mass spectrometry

NARI	Noradrenaline reuptake inhibitor
NBD	Nucleotide binding domain
NET	Noradrenaline transporter
NMR	Nuclear magnetic resonance
NSS	Neurotransmitter:sodium symporter
OAT	Organic anion transporter
PDB	Protein data bank
PEPT	Dipeptide transporter
PEPT1	H^+/dipeptide symporter
PfHT	Parasite-encoded facilitative hexose transporter
RMSD	Root mean square difference
RNA	Ribonucleic acid
RND	Resistance-nodulation-cell division
SERT	Serotonin transporter
SMR	Small multidrug resistance
SSRI	Selective serotonin reuptake inhibitor
TC	Transporter classification
TCDB	Transport classification database
TMD	Trans-membrane domain
TMH	Trans-membrane helix
VMAT	Vesicular monoamine transporter

1 Introduction

A number of consortia bringing together researchers from academic research institutions and companies have been established to determine the three-dimensional (3D) structures of proteins, rapidly and cost-effectively using modern methodologies [1]. At the end of April 2007, the number of entities in the PDB database (http://www.rcsb.org/pdb/) was greater than 42 000. The number of entities in the PDB database increased by more than 5000 during 2006, which is equivalent to the total number of entities in the database 10 years ago. Genome sequencing together with significant advances in process automation and informatics have aided the development of high-throughput X-ray crystallography, and are the main reasons for the large increase in the number of available 3D structures.

Atomic-resolution 3D structures provide important knowledge on biologically active molecules. The molecular structure of a protein contains information about the active site architecture, possible ligand or antigen binding sites, and evolutionary relationships within the protein family, and may serve as a basis for designing protein engineering experiments. The shape and electrostatic properties obtained from the molecular structure are also important for predicting possible interaction partners involved in regulation and complexation. Knowledge of the 3D structures of drug-target complexes defines the topography of the complementary surface between the drug target

and the ligands, and provides the possibility of virtual screening experiments searching for possible new molecules binding to the target [2] and for structure-aided drug design [3].

When detailed structural data for the target protein are available, computer programs can be used for ligand docking and virtual screening of compound libraries, and to predict protein–ligand binding affinities in the search for possible lead compounds. The obtained information can help the synthetic chemist to optimize compounds by including chemical groups that can form better interactions with the target, resulting in improved potency and selectivity [4]. At the moment there are several drugs on the market originating from a structure-based design approach. Examples include the HIV drugs Agenerase and Viracept developed using the X-ray crystal structure of HIV proteinase [5, 6], development of the flu drug Zanamivir based on the X-ray structure of neuraminidase [7], and the angiotensin-converting enzyme inhibitors [8, 9]. Direct structural determination by experimental methods like NMR spectroscopy and X-ray crystallography, and indirect structural knowledge obtained by different biophysical and molecular biology studies, together with bioinformatics and computational chemistry are of pivotal importance in the discovery and development of biologically active molecules, and of more effective and safer drugs.

During the last few years progress in genome sequencing has provided, and still provides, important information about the genetic map of different organisms. Modern technologies, such as microarray technology and 2D electrophoresis/mass spectrometry (MS), have provided insight into regulatory mechanisms at the DNA, RNA and protein levels. In the post-genomic era, focus will be on understanding the cellular machinery for regulation and communication, and how proteins and other gene products cooperate on a detailed atomic level. Such information provides insight into biological mechanisms and disease processes, and is important for the discovery and development of new drugs. However, knowledge of the detailed 3D structure of molecules involved in cellular communication will also be important in order to understand the cellular machinery. Structural information about central macromolecules and their regulation and interaction partners will most probably contribute to the discovery of new targets for therapeutic intervention, and may also give new insight into how drug targets can be therapeutically exploited. The drugs of the future may not only be traditional ligands functioning as an agonist, antagonist, substrate or inhibitor, but also act as scaffolding ligands by promoting protein–protein association, by preventing protein–protein association, or by enhancing or preventing degradation, internalization, etc. Future drugs may even be able to interfere with the specific signalling pathway(s) of a receptor without interfering with the other pathway(s) of the same receptor. Structural biology techniques, including theoretical calculations, and 3D structural information may therefore become even more important in the future.

Membrane transporter proteins are crucial co-players in cellular processes, and are known molecular components of many disease processes. The membrane transporter proteins are targeted by several presently used drugs, and have a large potential as targets for new drug development. In this review we discuss the current structural knowledge of membrane transporter proteins and its impact on new drug discovery.

2 Membrane Protein Structures

The protein targets for drug action on mammalian cells can broadly be divided into four main types: receptors, enzymes, ion channels and transporters. Integral membrane proteins are involved in a variety of processes governing cellular functions, and provide a plethora of molecular targets for pharmacological intervention. A large number (60–70%) of the presently known drug targets are proteins embedded in a cellular membrane, and membrane proteins are among the most interesting macromolecules to study by structural biology techniques. High-resolution structural information about proteins embedded in a cellular membrane is of pivotal importance for developing new drugs with therapeutic potential, but is also important for the understanding of the molecular mechanisms of cellular communication and function.

During the last few years, several international structural genomics networks have been established focusing on whole genomes [10], and some networks are focusing uniquely on membrane proteins. One of these networks is the EU-funded E-MeP consortium (http://www.ebi.ac.uk/e-mep/) that was established in 2005 with the goal of developing novel technologies to facilitate the purification and crystallization of membrane proteins. Currently around 20 European laboratories are members of the consortium, while additional laboratories are associate members. E-MeP is exploring several expression systems for 100 different prokaryotic and 200 different eukaryotic membrane proteins.

Crystallization and structure determination of membrane proteins are still not straightforward processes, and current knowledge of the detailed 3D structures of membrane proteins is limited. Out of the more than 42 000 entities deposited in the PDB database, only around 0.3% are unique structures of membrane proteins, although membrane proteins are estimated to represent approximately one third of the proteins coded for in the human and other genomes [11, 12]. Some of the most important questions in the fields of biology, chemistry and medicine remain unsolved as a result of the currently limited understanding of the structure, behaviour and molecular interactions of membrane proteins.

Integral membrane proteins of known 3D structure basically have two different types of architecture: α-helical bundles or β-barrels. Up to now,

eukaryotic plasma and reticulum membrane proteins have been shown to be α-helical, while the β-barrel membrane proteins are mainly found in the outer membrane of Gram-negative bacteria and in mitochondria and chloroplast membranes [13]. The helix bundle proteins contain quite long transmembrane hydrophobic α-helices that are packed together into bundles with relatively complicated structure, while the β-barrel proteins are large proteins consisting of anti-parallel β-sheets that fold into a barrel closed by the first and last strands of the sheet [14, 15]. In amino acid sequences of proteins with unknown 3D structure and function, the long hydrophobic transmembrane α-helices are easier to recognize in the sequence than the less hydrophobic trans-membrane β-strands. Bioinformatics studies are therefore generally easier to perform for α-helical bundle trans-membrane proteins than for β-barrel trans-membrane proteins, and have produced much more information about α-helical bundle proteins. Since the 3D structure of integral membrane proteins is not easily determined experimentally, prediction of the secondary structure from the amino acid sequence is important for annotating protein sequences to membrane protein families. This, together with recognition of structural motifs by bioinformatics, provides structural information of value for determining the function and predicting the 3D structure of trans-membrane proteins [16–18].

3 Membrane Transporter Proteins

Ions and small organic molecules are often too polar to penetrate the cellular membrane on their own, and require a transport protein. Trans-membrane solute transporters may be divided into channels that function as selective pores opening in response to a chemical or electrophysiological stimulus, thus allowing movement of a solute down an electrochemical gradient, and active carrier proteins which use an energy-producing process to translocate a substrate against a concentration gradient [19].

Transporter proteins have a recognition site making them specific for a particular solute. The human genome contains many different transporters, including those responsible for the transport of glucose and amino acids into cells, transport of ions and organic molecules by the renal tubules, transport of Ca^{2+} and Na^{+} out of cells, uptake of neurotransmitters and neurotransmitter precursors into nerve terminals and vesicles, and transporters involved in multidrug resistance. Drugs may exert their effect by binding to transporters and either inhibiting transport of the solute or functioning as a false substrate for the transport process.

Examples of such drugs include the antidepressant drugs that inhibit the neuronal transporters for noradrenaline and serotonin [20, 21], probenecid which inhibits the weak acid transporter protein in the renal tubule [22], loop

diuretics inhibiting the $Na^+/K^+/2Cl^-$ co-transporter of the loop of Henle [23], and the irreversible inhibitor of the H^+/K^+ ATPase (proton pump) of the gastric mucosa, omeprazole [24]. The lack of atomic-resolution 3D structures of membrane transporter proteins limits the design of new ligands interfering with the structure and function of the transporter. Only a few membrane transporter proteins from bacterial species have been crystallized and examined by X-ray diffraction experiments [25]. This makes molecular modelling by biocomputing an interesting methodological alternative, and in many cases the only method available for structural studies of membrane transporter proteins. However, such methods depend on a combination of computational techniques and experimental structural information to guide the molecular modelling process.

3.1 Classification of Membrane Transport Proteins

According to the classification approved by the transporter nomenclature panel of the International Union of Biochemistry and Molecular Biology [19], transporters belong to six categories:

1. Channels and pores
2. Electrochemical potential-driven transporters (secondary and tertiary transporters)
3. Primary active transporters
4. Group translocators
8. Accessory factors involved in transport
9. Incompletely characterized transport proteins

Categories 2, 3 and 4 are carriers. In contrast to most channels, carriers exhibit stereospecific substrate specificities, and their rates of transport are several orders of magnitude lower than those of other channels [19]. Mammalian species have carriers for peptides, nucleosides, sugars, bile acids, amino acids, organic anions, organic cations, vitamins, fatty acids, bicarbonate, phosphates and neurotransmitters. Numerous transporters of interest as drug targets belong to subclasses 2A (porters) and 3A (diphosphate bond hydrolysis-driven transporters).

Porters are either uniporters, symporters or antiporters. Uniporters are facilitated diffusion carriers that transport single molecules, symporters transport two or more molecules in the same direction, while antiporters transport two or more molecules in opposite directions [19].

Carrier mechanisms are distinguished by the source of energy used to activate the transporter, which may be either one of two:

- Facilitated diffusion
- Active transport

3.1.1 Facilitated Diffusion

Facilitated diffusion is accelerated by specific binding between the solute and the transporter. The solute flows from a higher to a lower electrochemical potential, so-called passive transport, via a uniporter, and facilitated diffusion therefore does not require a supply of energy. Examples of uniporters, or facilitated diffusion transporters, are glucose transporters (GLUTs), as indicated in Fig. 1, and the parasite-encoded facilitative hexose transporter (PfHT) of the major facilitator superfamily (MFS). Examples of GLUTs are GLUT1 and GLUT2. GLUT1 is expressed in highest concentrations in erythrocytes and in endothelial cells of barrier tissues, such as the blood–brain barrier. GLUT2 is expressed in liver cells, pancreatic beta-cells, renal tubular cells and intestinal epithelial cells that transport glucose. GLUT1 is responsible for the basal glucose uptake required to maintain respiration in all cells, and GLUT1 levels are decreased by increased glucose levels and increased by decreased glucose levels. PfHT is used by the malaria parasite to absorb glucose, which it needs to grow and multiply in red blood cells.

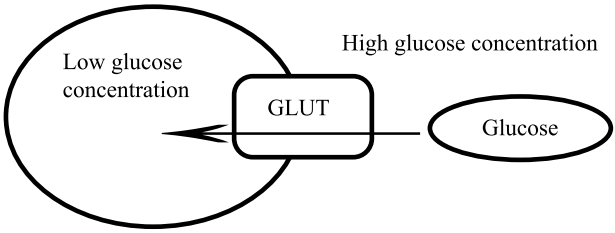

Fig. 1 Facilitated diffusion of glucose through GLUT down the concentration gradient

3.1.2 Active Transport Mechanisms

Active transport uses the free energy stored in the high-energy phosphate bonds of adenosine triphosphate (ATP) as energy source to activate the transporter. There are three types of active transport mechanisms: primary active transport, secondary active transport and tertiary active transport.

Primary active transporters (Fig. 2) use the energy from ATP directly. They exhibit ATPase activity to cleave ATP's terminal phosphate, and move substances from regions of low concentration to regions of high concentration. The ATP-binding cassette (ABC) transporters are primary active transporters comprising a family of structurally related membrane proteins that share a common intracellular structural motif in the domain that binds and hydrolyses ATP. ABC transporters are molecular pumps that regulate the movement of diverse molecules across cellular membranes and represent an

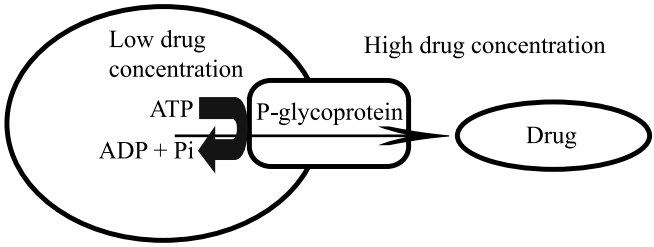

Fig. 2 Primary active transport of drug via P-glycoprotein. The energy from ATP is used to expel the drug out of the cell

important class of targets for discovery of novel small-molecule drugs for treatment of a broad range of human diseases. ABC transporters have both trans-membrane domains (TMDs) and nucleotide binding domains (NBDs). The domain arrangement of these transporters is generally TMD-NBD-TMD-NBD, but domain arrangements such as TMD-TMD-NBD-TMD-NBD, NBD-TMD-NBD-TMD, TMD-NBD and NBD-TMD have also been demonstrated [26, 27]. ABC transporters can be either exporters or importers. A well-characterized ABC exporter is P-glycoprotein, or ABCB1, which is widely distributed in normal cells, such as liver cells, renal proximal tubular cells, cells lining the intestine and the capillary endothelial cells of the blood–brain barrier. P-glycoprotein has broad substrate specificity and may have evolved as a defence mechanism against toxic substances. It actively pumps chemotherapeutic agents out of cancer cells, resulting in multidrug resistance to such drugs (Fig. 2).

Secondary active transporters (Fig. 3) use the energy from a concentration gradient previously established by a primary active transport process. Thus, secondary active transport indirectly uses the energy derived from the hydrolysis of ATP. The driving force of secondary active transport is an ion, for instance H^+ or Na^+, transported down its concentration gradient. Simultaneously, a substrate is transported against its concentration gradient.

There are two types of secondary active transport processes: antiport and symport. In antiport, the driving force ion and the substrate are transported

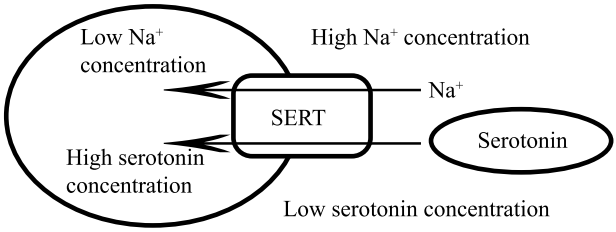

Fig. 3 Secondary active transport. The energy established by the Na^+ gradient is used to transport serotonin against its concentration gradient

in opposite directions, while in symport, they are transported in the same direction. Examples of secondary transporters are the H^+/dipeptide symporter (PEPT1) mainly involved in absorption of di- and tripeptides across plasma membranes in the small intestine and kidney proximal tubules, and central nervous system (CNS) transporters such as the serotonin (5-HT) transporter (SERT), noradrenaline transporter (NET), dopamine transporter (DAT), GABA transporter (GAT) and excitatory amino acid (glutamate) transporter (EAAT). By pumping neurotransmitters back into presynaptic nerve terminals, these CNS transporters play central roles in maintaining the homeostasis of neutrotransmitter levels in neuronal synapses.

Tertiary active transporters like the organic anion transporters (OATs). Tertiary active transporters utilize a gradient generated by secondary active transport. OATs use the outwardly directed dicarboxylate gradient to move (exchange) the organic substrate into the cell. The dicarboxylate gradient is generated by the sodium dicarboxylate co-transporter (secondary active transporter) which is using the inwardly directed sodium gradient initially generated by the Na^+/K^+-ATPase (primary active transporter) [28].

4 Structure Determination of Membrane Proteins

Although structural determination of membrane proteins is not a trivial task, improvements in membrane protein molecular biology and biochemistry, technical advances in structural data collection, notably using synchrotron X-ray beamlines, and the availability of several sequenced genomes have contributed to progress in the number of trans-membrane proteins determined by X-ray crystallography [29–31]. The difficulties in experimental structure determination of trans-membrane proteins arise from their amphiphilic nature. The hydrophilic surfaces are exposed to the aqueous medium, while the hydrophobic surfaces interact with non-polar alkyl chains of phospholipids. The amphiphilic nature makes it difficult to obtain stable and homogeneous protein preparations, and during crystallization, crystal contacts are formed between hydrophilic and hydrophobic surfaces.

Key issues that need to be considered before the structure of a trans-membrane drug target can be determined are [10]:

- How to produce a sufficient amount of the membrane protein.
- How to solubilize and purify the membrane protein without destroying the active 3D conformation of the protein. For membrane transporter proteins this is not trivial, due to the hydrophobic nature of the membrane-spanning region of the protein.
- How to crystallize the membrane transport protein, and what can be done in order to study the 3D membrane protein structure in solution.

4.1 Expression and Purification of Membrane Proteins

In order to determine a protein structure at high resolution, at least milligram quantities of the protein are required. In spite of recombinant protein production techniques and a variety of available expression systems, it has been difficult to provide membrane proteins in a quantity and quality for X-ray crystallographic structure determination. Membrane proteins are often expressed in low abundance in native tissues, and it is therefore necessary to produce the proteins in heterologous expression systems. However, heterologous membrane protein expression may produce toxic effects on host cells, contributing to poor stability and low yields [1]. This problem can be reduced by introducing deletions and mutations into the proteins and by generating fusion constructs. It is also important to use an expression system that does not significantly affect the activity of the mammalian membrane protein, compared with the activity in the native tissue [10]. Prokaryotes may lack many post-translational modification systems of importance for the native activity of the membrane protein. Many different types of recombinant expression systems have been tested for membrane proteins.

The most widely used system for recombinant protein expression of transmembrane proteins has been *Escherichia coli*, due to the simple and inexpensive scale-up [32], which has so far also been the most successful approach. The expression has been directed to the bacterial membrane or inclusion bodies. Suitable expression vectors are available, and proteins can be labelled metabolically with heavy-atom-labelled amino acids for X-ray crystallography or with stable isotopes for NMR spectroscopy [33]. In addition to *E. coli*, other bacteria have also been tested for membrane protein expression, but have usually given lower yields [34].

Different yeast strains have been used for recombinant expression of a number of trans-membrane proteins [10]. Insect cells have a close resemblance to mammalian cells and have been used for membrane protein expression [10, 35]. Expression in mammalian cells has also been performed, resulting in both transient and stable expression. A general drawback with the use of mammalian cell lines has been that it has given quite low yields compared with bacterial expression systems, and it also involves a more time-consuming procedure [36]. Expression in COS cells and HEK293 cells has successfully been done for membrane transporter proteins including the glutamate transporter [37, 38].

After expression, the protein is solubilized and separated from the lipid components by the use of detergents. This process very often requires an intensive screening process, since different detergents have to be used for different trans-membrane proteins [10]. After solubilization, the recombinant protein is often purified by affinity chromography methods, after insertion of histidine tags into the N- or C-terminal of the protein.

4.2 Structure Determination of Membrane Proteins

The methods used to determine high-resolution atomic structures of proteins are nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography. Structural determination by X-ray crystallography is so far the method with largest success for trans-membrane proteins. X-ray crystallography and NMR have complementary features in elucidating the structure–functional relationships of proteins and protein–ligand complexes. If a protein forms suitable crystals, X-ray crystallography may represent a convenient and rapid approach, while NMR spectroscopy may have advantages when the structure is partly distorted, exists in several stable conformations in solution or does not crystallize. Solution and solid-phase NMR are also alternatives for structure determination, especially for smaller proteins, but also for protein domains where the electron density is not observed by X-ray crystallography. This is exemplified by the solution NMR structure of the periplasmic signalling domains of the TonB-dependent outer membrane transporter FecA from *E. coli* [39]. Electron cryomicroscopy also contributes valuable structural information about membrane proteins, although at much lower resolution than that obtained by X-ray crystallography [40].

Since structure determination of membrane proteins by experimental methods has so far proven very challenging, structure prediction by homology modelling [25] using modern bioinformatics techniques may represent an alternative, and very often the only alternative, to obtain insight into the atomic structure of membrane transporters and other membrane proteins.

4.2.1 X-Ray Crystallography

The use of advanced protein expression and purification procedures, crystallization robots and powerful synchrotron radiation sources has enabled high-throughput structure determination using X-ray crystallographic techniques. Crystallization techniques and structure determination have become "high-throughput" for several protein families, but for membrane proteins including transporter proteins, the available crystallization and structure solution methods are not regarded as high throughput.

A high-resolution X-ray crystallographic structure provides structural information at an atomic level and is a powerful method for studying the structure of drug targets and their ligands. X-ray crystal structures represent time and space averages of all atoms present within the protein molecule, and may also provide information about the structural movements of the protein. The process of X-ray structure determination of trans-membrane proteins has different steps including crystallization of the purified membrane pro-

tein, measurements of crystal diffractions, calculation of electron density and model building [1, 10].

A major challenge of X-ray crystallography of trans-membrane proteins is to obtain suitable 3D crystals. Homogeneity and stability at high protein concentrations are important to obtain good results. Different strategies have been used for producing suitable crystals. These strategies include the use of detergents that replace the native membrane lipids and form mixed detergent–membrane protein micelles, crystallization using vapour diffusion, and crystallization using lipid cubic phases and bicelles [29]. The rationale behind the methods using cubic phases [41–43] or bicelles [44, 45] is that the solubilized membrane protein is inserted into a native-like environment that is believed to improve the chances of crystallization.

4.2.2 NMR Spectroscopy

NMR spectroscopy investigates transition between spin states of magnetically active nuclei in a magnetic field. Determination of the solution structure of trans-membrane proteins by NMR requires that well-resolved 2D $^{1}H/^{15}N$ chemical shift correlation spectra can be obtained. For helical transmembrane proteins, spectral resolution is complicated by the limited amide ^{1}H chemical shift dispersion in α-helixes and the slow correlation time for many micelle-bound proteins [46].

In general, NMR methods have advanced to the point where small to medium sized protein domain structures can be determined in a quite routine manner, and solution NMR spectroscopy has emerged as an eminent tool in studies of protein structure [47] and intermolecular interactions [48]. Of about 42 000 entities (April 2007) deposited in the PDB database (http://www.rcsb.org/pdb/), about 15% were determined by NMR techniques. NMR spectroscopy of proteins contributes with important information about the kinetics, thermodynamics, conformational equilibria, molecular motions and ligand binding equilibria of the protein, since the signals observed in solution by NMR show the chemical properties of atomic nuclei, including the their relative motions [49].

If over-expressed proteins are inserted efficiently into membranes, they might also be studied by solid-state NMR spectroscopy without prior dissociation. When this method can handle larger proteins, the method holds promise for 3D structure determination of membrane proteins [50, 51].

4.2.3 Electron Microscopy

The basic idea of electron microscopic 3D structure determination is to produce 2D projection images (2D crystals) from a 3D object. These 2D projec-

tion images can then be used to reconstruct the 3D structure of the original object by applying back-projection algorithms [1]. The method can be used to study large macromolecular machines like the ribosome or spliceosome which undergo massive structural rearrangements [40]. A number of membrane proteins have been reconstituted to form 2D crystals. The quality of the diffraction in the best direction of optimum crystals typically ranges from about 6–7 Å resolution up to 3 Å. At around 6 Å resolution trans-membrane α-helices can be revealed [52], while at 3 Å the protein backbone and larger side chains can be modelled.

4.2.4 Three-Dimensional Structure Prediction

Comparative modelling or homology modelling can be used to generate 3D structural models of proteins with unknown structure [53]. In homology modelling or comparative modelling, molecular modelling techniques are used to construct 3D models of the protein of interest (the target protein) using structural information from a protein with known 3D structure (the template protein), based on a postulated structural conservation between the template and target proteins. The homology modelling approach is based on the observation that the 3D structure of homologous proteins is more conserved than the amino acid sequence. Combined with structural information from molecular biology studies (e.g. site-directed mutagenesis experiments) and ligand binding studies, homology modelling provides indirect structural knowledge about the target protein and its interactions with drugs and other interaction partners.

When the structural similarities between the target and the template protein are high, the homology modelling approach may give structural models of sufficient accuracy for virtual screening of compound libraries and target-based ligand design. The accuracy of a model constructed by homology modelling depends on the conservation of secondary structure between the template and the target [54]. Sequence similarities larger than 50% between the template and the target are assumed to produce quite accurate structural models. Sequence similarities of 50% are expected to give a root mean square difference (RMSD) of about 1 Å between the backbone atoms of the template structure and the model. However, even at an overall sequence identity of <20% between the template and the target, the active sites and the secondary structure elements necessary for building the protein scaffold may have very similar geometries [55].

Several automatic homology modelling methods are available on the internet [56–58]. Such automatic modelling methods may provide models of high accuracy when the structural conservation between the template and the target is high. The most important single determinant for the quality of the homology-based model is the accuracy of the amino acid sequence

alignment between the template and the target [54]. For membrane transporters, the sequence identity between a bacterial template and a modelled mammalian membrane transporter is often low, and the alignment often has to be manually adjusted based on experimental observations, particularly from site-directed mutagenesis experiments [59]. The interpretation of site-directed mutagenesis results is therefore very important in the process of modelling membrane proteins. Models with low sequence similarity to the template structure are valuable working tools for generating hypotheses about the structure and function of the target protein, for designing new experimental studies, and along with structural information would contribute value to ligand design.

5
Transporters of Known 3D Structure

Three-dimensional crystal structures of several bacterial transporters for organic molecules have been determined by X-ray crystallography at atomic resolution, as shown in Table 1.

5.1
The Major Facilitator Superfamily

The major facilitator superfamily (MFS) includes almost 4000 different transporter proteins. The MFS family members transport diverse substrates including sugars, polyols, drugs, neurotransmitters, Krebs cycle metabolites, phosphorylated glycolytic intermediates, amino acids, peptides, organic and inorganic anions and many more (http://www.tcdb.org/) [60–63]. These transporters function by uniport, symport or antiport mechanisms, and may possess either 12 [64], 14 [65] or 24 [66] trans-membrane helices (TMHs), with a common evolutionary ancestor [67]. Examples of human MFS transporters are glucose uniporters (GLUTs), the vesicular monoamine transporter 1 and 2 (VMAT1 and VMAT2), the thyroid hormone transporter (MCT8) and the organic anion transporter (OAT) family.

The 3D structures of three *E. coli* transporter proteins of the MFS family have been determined by X-ray crystallography at atomic resolution: EmrD [68] (Fig. 4a), GlpT [69] and LacY [70] (Fig. 4b), at 3.5, 3.3 and 3.5 Å, respectively. These structures indicate that MFS proteins with 12 TMHs share a common architecture of the membrane spanning region, organized in symmetrical N- and C-terminal domains each of six TMHs, with overall structural topologies resembling each other. TMHs 3, 6, 9 and 12 are facing away from the interior of the transporters [68–70] (Fig. 4). The orientations of the TMHs of EmrD are different from those of GlpT and LacY, presumably

Table 1 Transporters for organic substrates with 3D structure determined at atomic resolution by X-ray crystallography. TC: transporter classification number (http://www.tcdb.org/)

Name	Family/superfamily	Function	Organism	TC number	PDB ident.	Refs.
EmrD (hydrophobic uncoupler, e.g. CCCP, benzalkonium, SDS):H^+ antiporter	Major facilitator superfamily (MFS)	H^+ antiport (multiple drugs)	*Escherichia coli*	2.A.1.2.9	2gfp	[68]
GlpT (glycerol-P:Pi antiporter)	Major facilitator superfamily (MFS)	Pi/glycerol-3-phosphate antiport	*Escherichia coli*	2.A.1.4.3	1PW4	[69]
LacY (lactose:H^+ symporter)	Major facilitator superfamily (MFS)	Symport (lactose/H^+)	*Escherichia coli*	2.A.1.5.1	1PV6 1PV7	[70]
AcrB (multidrug/dye/detergent resistance pump)	Resistance-nodulation-cell division (RND) superfamily	H^+ antiport	*Escherichia coli*	2.A.6.2.2	1OY6 etc	[73]
EmrE (small multidrug efflux pump)	Drug/metabolite transporter (DMT) superfamily	H^+ antiport	*Escherichia coli*	2.A.7.1.3	1S7B 2F2M	[82]
$LeuT_{Aa}$ (amino acid (leucine):2 Na^+ symporter)	Neurotransmitter:sodium symporter (NSS) family	Na^+ symport	*Aquifex aeolicus*	2.A.22.4.2	2A65	[64]
Glt_{ph} (archaeal glutamate transporter homologue)	Dicarboxylate/amino acid:cation (Na^+ or H^+) symporter (DAACS) family	H^+ symport	*Pyrococcus horikoshii*	2.A.23.1.5	11XFH	[86]
Sav1866 (multidrug exporter)	ABC superfamily		*Staphylococcus aureus*	3.A.1.106.2	2HYD	[88]
BtuCD (bacterial ABC transporter involved in B_{12} uptake)	Vitamin B_{12} uptake transporter ($B_{12}T$) family (ABC superfamily)	Vitamin B_{12} uptake (ATP)	*Escherichia coli*	3.A.1.13.1	1L7V	[93]
HI1470/1 (bacterial ABC transporter involved in haem and B_{12} uptake)	Vitamin B_{12} uptake transporter ($B_{12}T$) family (ABC superfamily)	Haem and B_{12} uptake	*Haemophilus influenzae*	–	2NQ2	[94]

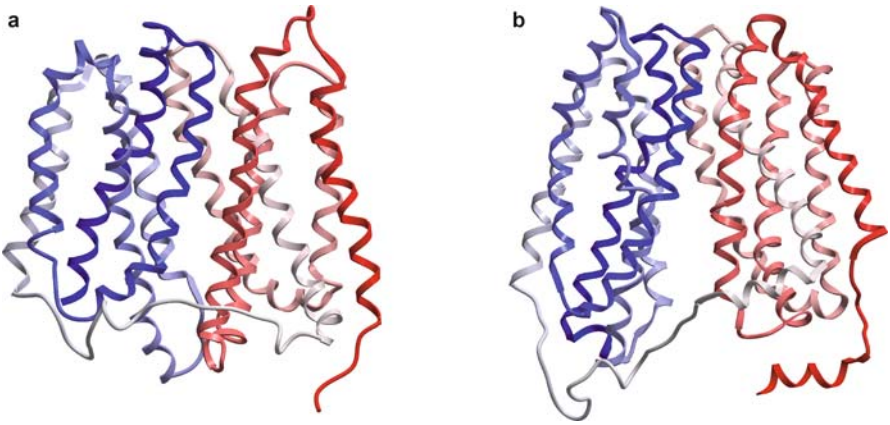

Fig. 4 Backbone Cα trace of the X-ray crystallographic structure of EmrD (**a**) [68] (PDB code 2GFP) and LacY (**b**) [70] (PDB code 1PV6), viewed in the membrane plane (cytoplasm downwards). *Colour coding* of the structures: *blue* via *white* to *red* from N-terminal to C-terminal

because GlpT and LacY are facing the cytoplasm in a V-shaped conformation [69, 70], while EmrD probably represents an intermediate state [68]. It has been proposed that the substrates for GlpT, LacY and EmrD are translocated across the membrane by an alternating-access mechanism [68–70].

5.2 The Resistance-Nodulation-Cell Division Superfamily

All known members of the resistance-nodulation-cell division (RND) superfamily catalyse substrate efflux via and H^+ antiporter mechanisms. These transporters are found in bacteria, archaea and eukaryotes and are organized in eight phylogenic families (http://www.tcdb.org/) [71, 72]. Up to now, 18 different X-ray crystallographic structures of the proton:drug antiporter Acriflavine resistance protein B (AcrB) have been reported [73–77] [78] (Fig. 5). This transporter is a major drug-resistance pump of the RND superfamily that belongs to the largely Gram-negative bacterial hydrophobe/amphiphile efflux-1 (HAE1) family (http://www.tcdb.org/). In *E. coli* the protein cooperates with a membrane fusion protein AcrA, and the outer membrane channel Tol C [73–75]. The substrate specificity of this large protein complex is broad, transporting cationic neutral and anionic substrates [79]. The AcrB protomer is organized as a homotrimer with a jellyfish-like structure [73], and the crystal structures of AcrB with and without substrates indicate that drugs are exported by a functionally rotating mechanism [74] or an alternating access peristaltic mechanism [75].

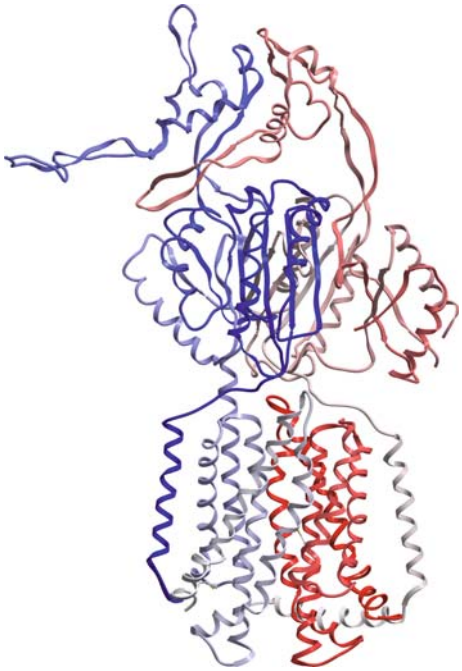

Fig. 5 Backbone Cα trace of the X-ray crystallographic structure of a single AcrB protomer [73] (PDB code 1IWG) viewed in the membrane plane. *Colour coding* as in Fig. 4

5.3 The Drug/Metabolite Transporter Superfamily

The drug/metabolite transporter (DMT) superfamily consists of 18 recognized families, each with a characteristic function, size and topology [80]. The multidrug transporter EmrE belongs to this superfamily, and to the four TMH small multidrug resistance (SMR) family (http://www.tcdb.org/). The SMR family members are prokaryotic transport systems consisting of homodimeric or heterodimeric structures [81]. Two *E. coli* EmrE X-ray crystal structures have been reported at 3.7 [82] and 3.8 Å [83]. The EmrE transporter is a proton drug:antiporter [82], and two EmrE subunits form a homodimer that binds substrate at the interface [83] (Fig. 6).

5.4 The Neurotransmitter:Sodium Symporter Family

Members of the neurotransmitter:sodium symporter (NSS) family catalyse uptake of a variety of neurotransmitters, amino acids, osmolytes and related nitrogenous substances by a solute:Na^+ symport mechanism [84]. In 2005 the crystal structure of the *Aquifex aeolicus* $LeuT_{Aa}$ determined by X-ray diffrac-

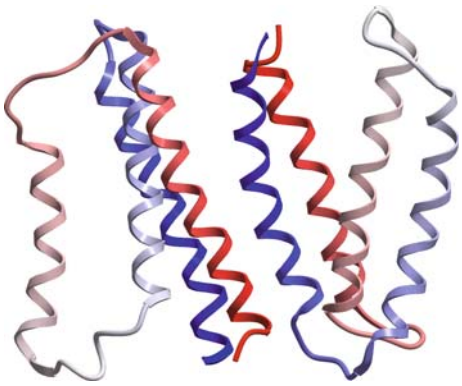

Fig. 6 Cα trace of the X-ray crystal structure of the EmrE dimer X [83] (PDB code 2F2M) viewed in the membrane plane. *Colour coding* as in Fig. 4

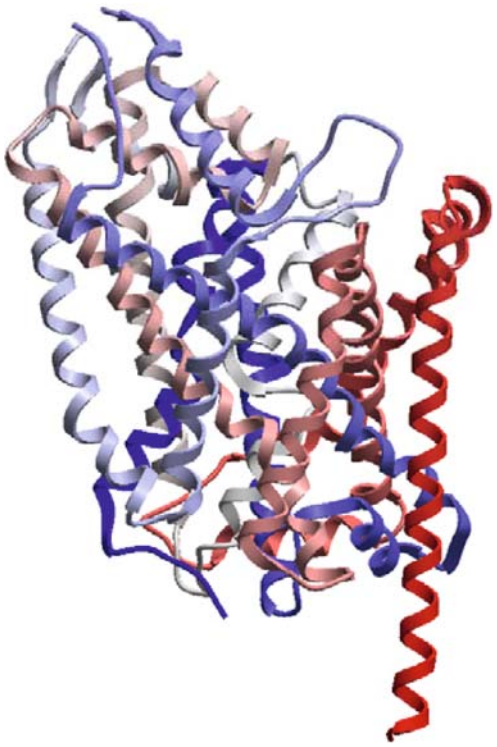

Fig. 7 Cα trace of the $LeuT_{Aa}$ X-ray crystallographic structure [64] (PDB code 2A65) viewed in the membrane plane. *Colour coding* as in Fig. 4

tion at 1.65 Å resolution was reported [64] (Fig. 7). $LeuT_{Aa}$ belongs to the NSS family (http://www.tcdb.org/), and is a bacterial homologue of SERT, DAT, NET and GAT-1. It is a 12 TMH sodium/leucine symporter, where TMHs 1–

5 are related to TMHs 6–10 by a pseudo-twofold axis in the membrane plane. The structure resembles a shallow "shot glass" [64], with leucine and sodium ions bound within the protein core. The substrate and sodium ion binding sites are comprised of TMHs 1, 3, 6 and 8. An alternating access model for transport, where all symported substrates must bind simultaneously before translocation, has been confirmed by experimental studies on SERT [85].

5.5 The Dicarboxylate/Amino Acid:Cation (Na^+ or H^+) Symporter Family

The members of the dicarboxylate/amino acid:cation symporter (DAACS) family catalyse Na^+ and/or H^+ symport together with either a Krebs cycle dicarboxylate (malate, succinate or fumarate), a dicarboxylic amino acid (glutamate or aspartate), a small, semipolar, neutral amino acid (Ala, Ser, Cys, Thr), neutral and acidic amino acids or most zwitterionic and dibasic amino acids (http://www.tcdb.org/).

The *Pyrococcus horikoshii* Glt_{ph} (archaeal glutamate transporter homologue) structure has been determined by X-ray crystallography at 3.5 Å resolution [86] (Fig. 8) and 2.96 Å resolution [87]. This is a proton symporter belonging to the DAACS family (http://www.tcdb.org/). The transporter is organized as a trimer, with each protomer having eight TMHs, two re-entrant helical hairpins, and independent substrate translocation pathways [87]. It has been proposed that glutamate transport is achieved by movements of the

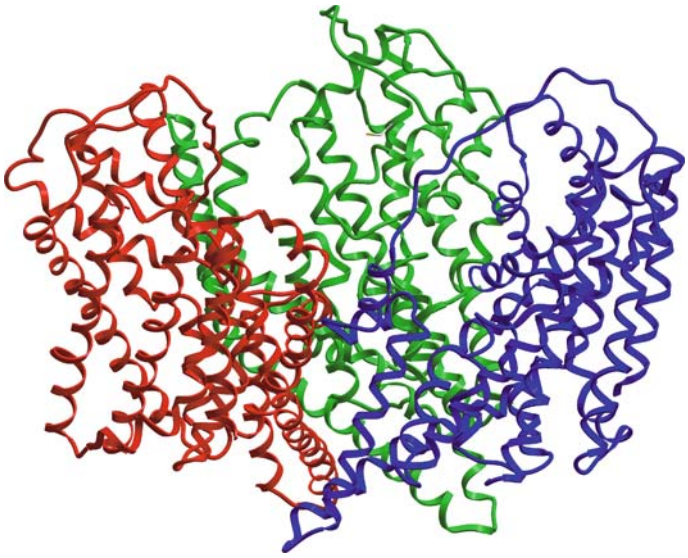

Fig. 8 Backbone Cα trace of the Glt_{ph} trimer [86] (PDB code 1XFH) viewed in the membrane plane. The three protein subunits are shown in different colours (*red*, *green*, *blue*)

hairpins allowing alternating access to either side of the membrane [86]. Glt_{ph} is a bacterial homologue of the human EAAT1–5.

5.6 The ATP-Binding Cassette Superfamily

The ABC superfamily contains both uptake and efflux transport systems. Phylogenically, the members of these two porter groups generally cluster loosely together with just a few exceptions. There are dozens of families within the ABC superfamily, and family classification generally correlates with substrate specificity (http://www.tcdb.org/).

In 2006, the multidrug transporter *Staphylococcus aureus* Sav1866 was determined by X-ray crystallography at 3.0 Å resolution in an outward-facing conformation, reflecting the ATP-bound state [88]. Sav1866 belongs to the ABC superfamily and shows sequence similarity to human P-glycoprotein (P-gp). The transporter consists of two subunits, each with a trans-membrane domain–nuclear binding domain (TMD-NBD) topology, with six TMHs in each TMD. The two subunits are twisted and embracing each other, and both the TMDs and NBDs are tightly interacting. Towards the extracellular side, bundles of TMHs diverge into two "wings", with each wing consisting of TMH1 and TMH2 from one subunit and TMH3–TMH6 from the other subunit. The crystal structure of Sav1866 indicates that ABC transporters may use an "alternating access and release" mechanism where ATP binding and hydrolysis control the conversion of one state into the other, and that domain swapping and subunit twisting takes place in the transport cycle [88].

The MsbA structures from three different bacteria have been determined by X-ray crystallography at 4.5 Å resolution (*E. coli*) [89], 3.8 Å resolution (*Vibrio cholerae*) [90], and 4.2 Å resolution (*Salmonella typhimurium*) [91]. The MsbA transporters belong to the ABC superfamily, and the prokaryotic ABC-type efflux permeases or the lipid exporter (LipidE) family (http://www.tcdb.org/). The three different structures were thought to represent different conformational stages of the transport cycle, an open conformation [89], a closed conformation [90], and a post-hydrolysis conformation [91]. If true, the MsbA structures would represent interesting targets for computer modelling of human ABC transporters. However, these structures were retracted [92] after the publication of the Sav1866 structure [88]. The Sav1866 structure indicated that the MsbA structures were incorrect and that the biological interpretations based on the MsbA structures were invalid.

The *E. coli* BtuCD protein is involved in B_{12} uptake, and the structure was determined by X-ray crystallography at 3.2 Å resolution [93]. BtuCD also belongs to the ABC superfamily, prokaryotic ABC-type uptake permeases, and the vitamin B_{12} uptake transporter ($B_{12}T$) family. It consists of four subunits, two NBDs (BtuD) and two TMDs (BtuC). Each of the two BtuC subunits contains ten TMHs [93].

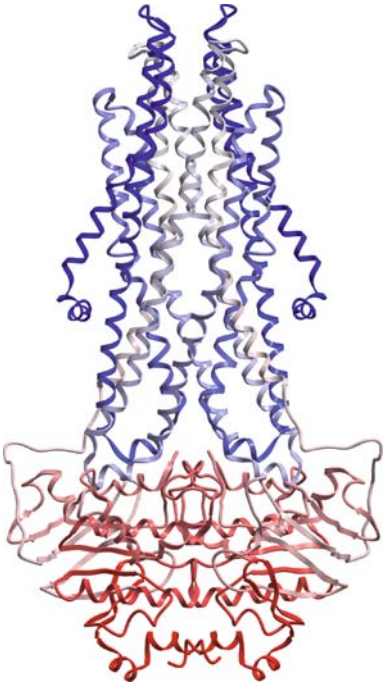

Fig. 9 Backbone Cα trace of the Sav1866 dimer [88] (PDB code 2HYD) viewed in the membrane plane. The NBDs are in *red*, while the TMDs are coloured in *blue* to *white* from the N- to the C-terminal

The 3D structure of the HI1470/1 transporter from *Haemophilus influenzae*, which is also a bacterial ABC transporter mediating the uptake of metal-chelate species including haem and vitamin B_{12}, has been determined at 2.4 Å resolution [94]. It exhibits an inward-facing conformation, which is in contrast to the outward-facing state observed for the homologous vitamin B_{12} importer BtuCD [94]. The 3D structures indicate that the substrate translocation requires large conformational changes, and the differences between the BtuCD and the HI1470/1 transporter from *H. influenzae* may reflect conformations relevant to the alternating access mechanism of substrate translocation.

6 Potential for New Drug Development

6.1 Multidrug Resistance Protein Targets

Cells exposed to toxic compounds can develop resistance by a number of mechanisms, including increased excretion. This may result in multidrug

resistance, which is a particular limitation to cancer chemotherapy and antibiotic treatment. Development of inhibitors of drug efflux transporters has been sought for use as a supplement to current therapy in order to overcome multidrug resistance problems [95].

6.1.1 ABC Transporters and Cancer Therapy

ABC transporters play an important role in multidrug resistance in cancer chemotherapy. Human ABC transporters are divided into five different subfamilies ABCA, ABCB, ABCC, ABCD and ABCG, based on phylogenetic analysis. According to the transport classification database (TCDB), these ABC transporter subfamilies (ABC-type efflux permeases) belong to subclasses 3.A.1.201–212 [19] (http://www.tcdb.org/). Transporters in subfamilies ABCA, ABCB, ABCC and ABCG are involved in multidrug resistance [96–99].

ABCB1 (P-Glycoprotein)

The ABCB1 transporter is important in the removal of anticancer agents, such as adriamycin, vincristine and daunorubicin, from cells. ABCB1 is expressed in normal tissues, such as the gastrointestinal epithelium, epithelia of the bronchi, mammary gland, prostate gland, salivary gland, sweat glands of the skin, pancreatic ducts, renal tubules, and in bile canaliculi and ductules, in adrenal and in endothelial cells at blood–brain barrier sites and other blood–tissue barrier sites [100]. ABCB1 expression is highest in tumours from colon, adrenal, pancreatic, mammary and renal tissue, even in the absence of prior chemotherapy [101]. Even though the relationship between ABCB1 expression and response to chemotherapy remains unclear, negative prognostic implications of ABCB1 expression have been established in breast cancer, neuroblastoma, various types of leukaemia, and several sarcomas [101].

Development of ABCB1 inhibitors may help to prevent ABCB1 efflux of anticancer agents. ABCB1 inhibitors are not cytotoxic agents themselves, but when used in combination with cancer drugs which are normally pumped out by the cell by ABCB1, intracellular drug concentrations are maintained, restoring sensitivity to these therapeutics.

Three generations of ABCB1 inhibitors have been developed. The first-generation ABCB1 inhibitors were established therapeutic drugs for diverse targets that were discovered, largely by chance, to also function as ABCB1 inhibitors [101]. In general, these were less potent than later generations of ABCB1 inhibitors; they were not selective, and produced undesirable side effects [95]. The second-generation ABCB1 inhibitors, which were based on the structures of the first-generation compounds and optimized using QSAR [101], were less toxic. However, inhibition of ABCB1 by first- or second-generation compounds has failed to demonstrate the desired clinical benefit.

Dangerously high doses of these agents were needed, and they exhibited toxicity due to an increased availability of the co-administered chemotherapy [95].

The third-generation ABCB1 inhibitors, which were discovered by combinatorial chemistry screening [101], are more potent and more selective than earlier compounds, and are currently in clinical trials [101, 102]. The therapeutic benefit of ABCB1 inhibition is yet to be firmly established, but the continued development of these agents may establish the true therapeutic potential of ABCB1-mediated multidrug resistance reversal. A suggested approach would achieve a balance between the positive effects of ABCB1 inhibition at the tumour site and the negative potential toxic side effects outcome of reducing elimination of the chemotherapy.

ABCC5 (MRP5)

ABCC5 belongs to the ABC superfamily, transports cGMP and is also involved in multidrug resistance [103]. ABCC5 is expressed in most tissues, such as in skeletal muscle, kidney, testis, heart and brain [104–106], in smooth muscle cells of the corpus cavernosum, ureter and bladder, and mucosa in ureter and urethra [107, 108], in vascular smooth muscle cells, cardiomyocytes, and vascular endothelial cells in the heart [109], in placenta [110], and in human erythrocytes [103]. Clinical studies have shown that extracellular cGMP levels are elevated in various types of cancer. Significant elevation of urinary cGMP excretion has been observed in patients with untreatable adenocarcinomas from ovary, stomach or large bowel, whereas a normal range was found in patients where tumours had been removed [111]. Elevated urine cGMP concentrations have also been demonstrated among patients with cancer of the uterine cervix [112], and measurements of urinary cGMP levels after treatment of ovarian cancer has been reported to be a very sensitive tool in therapeutic monitoring [113–115]. Increased cGMP efflux by ABCC5 may be one mechanism whereby cancer cells can develop resistance against endogenous growth control, and also against antineoplastic drugs which are substrates for ABCC5.

ABCB1/ABCC5 Structural Considerations/Molecular Modelling Approach

Knowledge of the ABCB1 and ABCC5 structures may be used to develop membrane transport modulating agents which, in turn, may be helpful in overcoming resistance to chemotherapeutic agents. These transporters feature both TMDs and NBDs, with a TMD-NBD-TMD-NBD domain arrangement. The NBD contains the Walker A and B motifs [116] and a signature C motif, and the substrate specificity of the transporters is provided by the TMDs.

The 3D structures of ABCB1 and ABCC5 have not been experimentally determined, but molecular modelling by homology may be used to gain

structural insight into their potential as drug targets. In particular, homology modelling may be used to study substrate difference between ABCB1 and ABCC5, since ABCB1 transports cationic amphiphilic and lipophilic substrates [117–120], while ABCC5 transports organic anions [103, 121].

In order to understand the molecular concepts underlying the substrate difference between ABCB1 and ABCC5, we have used the *Staphylococcus aureus* Sav1866 X-ray crystal structure [88] to construct models of ABCB1 and ABCC5 [122]. Modelling indicated that the electrostatic potential surface of the substrate translocation area of ABCB1 is neutral with negative and weakly positive areas, while the electrostatic potential surface of the ABCC5 substrate translocation chamber generally is positive. These results indicate that ABCB1, transporting cationic amphiphilic and lipophilic substrates, has a more neutral substrate translocation chamber than ABCC5, which has a positive chamber transporting organic anions. Structural information about the ABCB1 and ABCC5 substrate binding sites might be useful in the design of inhibitor multidrug efflux by these transporters.

6.2 Multidrug Resistance and Antibiotic Treatment

Treatment of infections may be limited by the emergence of bacteria that are resistant to multiple antibiotics. Bacterial antibiotic resistance may be caused by intrinsic mechanisms, such as efflux systems, or by acquired mechanisms, such as mutations in genes targeted by the antibiotic [123].

The major mechanism of resistance to tetracycline in Gram-negative bacteria is drug-specific efflux. Drug efflux pumps are involved in fluoroquinolone resistance of *Staphylococcus aureus* and *Streptococcus pneumoniae*, and the antiseptic resistance of *Staphylococcus aureus*. When multidrug pumps are overexpressed, resistance levels are elevated. Efflux pumps are thus potential antibacterial targets, since inhibitors of bacterial efflux pumps may restore the activity of an antibiotic which otherwise is effluxed [124]. Structural knowledge at the atomic level from X-ray crystallographic studies of bacterial multidrug transporters is rapidly growing. Examples are the *E. coli* EmrD [68], *E. coli* AcrB [73–78], *E. coli* EmrE [82, 83], and *Staphylococcus aureus* Sav1866 [88] crystal structures.

6.3 CNS Drug Targets

6.3.1 Neurotransmitter:Sodium Symporter Family

Some of the most successful CNS drugs selectively target secondary transporters. Transporters at the plasma membrane contribute to the clearance

and recycling of neurotransmitters in neural synaptic clefts. When a neurotransmitter transporter is inhibited, the concentration of neurotransmitter increases in the synapse. The *A. aeolicus* $LeuT_{Aa}$ crystal structure [64] of the NSS family has delivered new insight into the structure of NSS transporters. Its homologies to the human transporters SERT, NET and DAT are 20–25% [64]. The high sequence conservation in functionally important regions between the *A. aeolicus* $LeuT_{Aa}$ transporter and the other NSS family members suggests that these proteins share a common folding and a common transport mechanism, and that the *A. aeolicus* $LeuT_{Aa}$ crystal structure can be used to model the functionally important regions of other NSS family members with quite high accuracy [59].

Serotonin and Noradrenaline Transporters

Noradrenaline and 5-HT modulate the activity of neural circuits influencing mood and sleep. Antidepressants selectively inhibit 5-HT or noradrenaline reuptake into presynaptic neurons. Selective serotonin reuptake inhibitors (SSRIs) have replaced tricyclic antidepressants as the drugs of choice in the treatment of depressive disorders, mainly because of their improved tolerability and safety if taken in overdose. Still, 10–30% of patients taking antidepressants are partially or totally resistant to the treatment. SSRIs block the reuptake of serotonin into the presynaptic nerve terminals, thereby enhancing serotonergic neurotransmission, which presumably results in their antidepressant effects. SSRIs are prescribed for conditions such as depression, obsessive-compulsive disorder, social phobia, post-traumatic stress disorder, premenstrual dysphoric disorder and generalized anxiety disorder [125]. Side effects of SSRIs include agitation, insomnia, neuromuscular restlessness, nausea, dry mouth, fatigue, decreased libido, diarrhoea, vomiting and headache.

Reboxetine is a specific noradrenaline reuptake inhibitor (NARI). The side effects of NARIs include dry mouth, constipation, insomnia, increased sweating, tachycardia, vertigo, urinary retention and impotence. The general limitations of SSRIs and NARIs are due to side effects directly related to their effect on the serotonergic and noradrenergic systems. Discontinuation symptoms from SSRIs and NARIs are depression, dizziness, nausea, lethargy, headache, flu-like feelings, panic attacks, numbness, agitation and insomnia. A better understanding of the molecular mechanisms of SERT and NET is important for developing new agents with fewer side effects.

The molecular aspects of SSRI binding to SERT and NET have been the subject of several molecular modelling studies [126–129]. The *A. aeolicus* $LeuT_{Aa}$ crystal structure [64] represented a major advance towards understanding the structure–function relationships of SERT and NET, since this transporter is quite close both in function and amino acid sequence to human SERT and NET, and thus provides a template for updated models [59, 85, 130, 131]. In order to examine the molecular aspects of the selectivities of SSRIs,

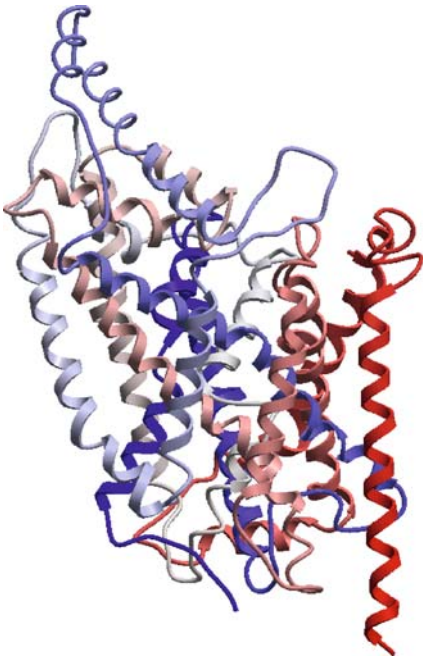

Fig. 10 Cα trace of the homology model of SERT [131] viewed in the membrane plane. *Colour coding* as in Fig. 4

we have constructed molecular models of SERT [131] (Fig. 10) and NET based on the *A. aeolicus* $LeuT_{Aa}$ crystal structure [64]. The ICM pocket finder of the ICM software version 3.4–4 [132] reported amino acids in TMHs 1, 3, 6 and 8 of SERT and NET as being contributors to the putative substrate binding area.

Dopamine Transporter and Drugs of Abuse

Dopamine is involved in the reward system, which is linked to drug abuse. When a person receives positive reinforcement for certain behaviours, which can be both natural rewards and artificial rewards such as addictive drugs, the reward system is activated [133]. When cocaine binds to the dopamine transporter (DAT), the dopamine concentration at the synapse is elevated, resulting in activation of a "reward" mechanism. The binding of cocaine to SERT and NET also contributes to cocaine reward and cocaine aversion [134, 135].

We have previously constructed 3D models of DAT [127–129, 136] based on various low-resolution structural data and transporters with low homology with DAT. The *A. aeolicus* $LeuT_{Aa}$ X-ray crystal structure [64] provides the possibility of updating the previous DAT models. Figure 11 shows a putative binding site of cocaine in DAT (unpublished). Site-directed mutagenesis studies and docking studies of cocaine binding to DAT indicated that cocaine

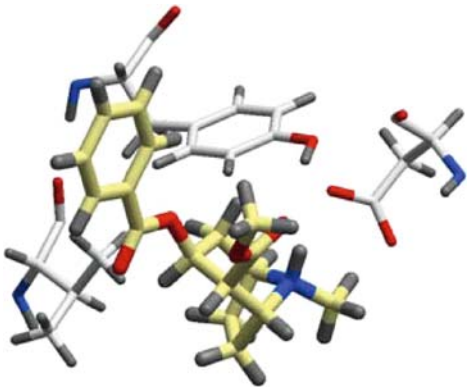

Fig. 11 Cocaine docked into the putative binding site of the DAT model. Amino acids interacting with cocaine displayed in the figure are Asp-79, Val-152 and Tyr-156

interacts with Asp-79 [137], Val-152 [138] and Tyr-156. Tyr-156 corresponds to Tyr-176 in SERT, which has been found by site-directed mutagenesis studies to be important for cocaine binding [139].

Interestingly, cocaine and SSRIs have similar molecular mechanisms of action. However, while SSRIs are therapeutic drugs prescribed for the treatment of depression, cocaine is a local anaesthetic drug and a substance of abuse. Knowledge of cocaine's molecular interactions with DAT may be used to develop agents that block binding of cocaine without inhibiting the reuptake of dopamine. Such agents might be effective in treating cocaine addiction.

GABA Transporter

The neurotransmitter GABA transmits inhibitory signals that reduce excitation and anxiety. A search for selective inhibitors of GABA transporters has led to potent and selective inhibitors of GAT-1 (SwissProt accession number P30531), which is a 12 TMH transporter with homology to $LeuT_{Aa}$ [59]. The only clinically approved GAT-1 inhibitor at present is tiagabine [140, 141]. Tiagabine is a potent and broad spectrum anticonvulsant drug which does not induce tolerance to the anticonvulsant effect [142]. Tiagabine has also shown promise in clinical trials to treat chronic daily headaches with symptoms of migraine [143], and it has been suggested from preclinical studies and human studies that tiagabine also possesses anxiolytic properties [141]. Tiagabine has also been reported to be effective in prophylactic treatment of bipolar disorder [144, 145], but its therapeutic potential in this condition has not been established.

A comprehensive amino acid alignment, including the *A. aeolicus* $LeuT_{Aa}$ sequence, sequences of GABA transporters and the sequences of other NSS family members, would provide the possibility of modelling the ligand bind-

ing to GAT-1 and GAT-2, which could be helpful in designing tiagabine derivatives with an improved binding selectivity profile.

Glycine Transporter

Non-competitive *N*-methyl-D-aspartate (NMDA) blockers induce schizophrenic-like symptoms in humans, presumably by impairing glutamatergic transmission [146]. It has therefore been postulated that compounds potentiating this neurotransmission would increase extracellular levels of glycine, which is a co-agonist of glutamate, and thus possess antipsychotic activity [146, 147].

GlyT1c is a glycine/$2Na^+$/$1Cl^-$ symporter belonging to the NSS family. Blocking of the GlyT1c transporter using a specific inhibitor, SSR504734, resulted in increased extracellular glycine levels in rat prefrontal cortex, and enhanced glutamatergic neurotransmission. The GlyT1c inhibitor has shown activity in several animal models of schizophrenia [146]. A homology model based on the $LeuT_{Aa}$ template will provide the possibility to study the molecular interactions of specific inhibition by SSR504734.

6.3.2 The Drug:H^+ Antiporter-1 (DHA1) (12 Spanner) Family

The vesicular monoamine transporter-2 (VMAT2) contributes to regulation of the monoaminergic neuronal function by sequestrating catecholamines and serotonin into synaptic vesicles. VMAT2 pumps dopamine, serotonin, noradrenaline, epinephrine and histamine into storage vesicles against a gradient, powered by the vesicular H-ATPase and the exchange of two protons for one substrate molecule [148]. These transporters also sequester neurotoxins within vesicles, thus playing a role in neuroprotection [149, 150].

Experimental studies have indicated that amphetamine-type agents interact with the VMAT2 and deplete vesicular neurotransmitters via a carrier-mediated exchange mechanism [151]. The cytoplasmic levels of transmitter are increased due to disruption of the vesicular transmitter storage of transmitter, such that more neurotransmitter is available for release by transporter-mediated exchange [151]. It has been proposed, based on its functions as a critical regulator of neurotransmitter disposition within the brain, that the VMAT2 transporter might be a possible target for drugs used in addictive disorders, Parkinson's disease and schizophrenia [152, 153].

VMAT2 is a member of the MFS family of proteins. Three different *E. coli* transporter proteins of the MFS family have been determined by X-ray crystallography at atomic resolution: EmrD [68] (Fig. 4a), GlpT [69], and LacY [70] (Fig. 4b). These structures indicate that the MFS proteins with 12 TMHs share a common architecture of the membrane spanning region, organized in symmetrical N- and C-terminal domains each of six TMHs, with overall structural topologies resembling each other. These observations indi-

cate that the MFS proteins of known 3D structure can be used to predict the structure of VMAT2, and thereby guide the synthesis and evaluation of novel VMAT2 ligands as possible therapeutic agents.

6.3.3
The Dicarboxylate/Amino Acid:Cation (Na^+ or H^+) Symporter Family

Glutamate Transporters

Glutamate causes excitation, nudging the brain into high alert, affecting cognition and most other fundamental aspects of brain function. It transmits critical instructions between nerve cells involved in sensory perception, learning and memory. Changes in glutamate neurotransmission may contribute to different brain diseases. Glutamate has a dual action: it is an excitatory neurotransmitter under normal conditions, but it is toxic to neuronal cells when present in excess. Selective modulation of the levels of glutamate may produce a therapeutic benefit in disorders where glutamate levels are abnormal, such as in stroke, head trauma, retinal ischaemia, schizophrenia, Alzheimer's disease, Parkinson's disease and other neurodegenerative and psychiatric disorders.

The excitatory amino acid transporter-3 (EAAT-3) is a major neuronal transporter for glutamate in the brain. An EAAT-3 inhibitor may have therapeutic applications in schizophrenia, cognitive impairment (such as that associated with Alzheimer's disease) and other nervous system diseases where glutamate neurotransmission is deficient (http://www.neurocrine.com/html/res_eaats.htm). Increasing the amount of glutamate released from certain nerve cells could improve learning, memory skills and overall cognitive function. A possible limitation to this approach is that glutamate can cause glutamate-induced retinal toxicity, and that long-term inhibition of glutamate transporter activity may cause neuronal damage. Increased knowledge of the structure and function of glutamate transporters is therefore of pivotal importance. So far, the only X-ray crystal structure with similarities in function and sequence with the glutamate transporters is that of the glutamate transporter homologue from *Pyrococcus horikoshii* Glt_{ph} [86].

Sequence alignment of family members followed by homology modelling should take into account that indirect structural knowledge from experimental studies may emphasize the nature of the ligand binding area of the EAAT-3 transporter.

6.4
Transporters Involved in Drug Absorption, Distribution and Elimination

More than 28 different mammalian OATs are expressed in the liver, small intestine, blood–brain barrier endothelial cells, placenta, kidneys and other

organs [154, 155]. These carrier proteins, which may transport a wide range of substrates, are major factors in drug absorption, distribution and excretion, and work in concert with the drug metabolism system in order to eliminate drug metabolites from systemic circulation. Their activity is saturable and inducible, and may show polymorphic variation among individuals [156].

P-glycoproteins, Mrps and Oatps/OATPs are transporter proteins playing an important role in drug absorption, distribution and excretion [154, 155, 157]. The organ-specific cellular localization and expression of such transporter proteins in liver, kidneys and intestines has given new insight into the molecular mechanisms of cellular uptake and excretion of drugs and drug metabolites in these organs. Uptake of many different compounds into hepatocytes is mediated by OATs expressed at the sinusoidal membrane (rat Oatp1, Oatp2 and Oatp4, human OATP-B, OATP-C and OATP8) [155]. These Oatps/OATPs mediate sodium-independent uptake of a wide variety of mainly bulky organic anions, neutral compounds and organic cations including drugs [154, 157]. Besides liver specific Oatps/OATPs (Oatp4, OATP-C and OATP8), others are expressed in various tissues including intestine (Oatp3), kidney (Oatp1), brain (Oatp2, Oatp3, OATP-A, OATP-D, OATP-E and OATP-F) and testis (OATP-D and OATP-F) [154]. It has been proposed that Oatps/OATPs may be used to target drugs to certain organs, based on their selective tissue distribution and substrate specificities.

In the future, detailed knowledge of cellular transport mechanisms of drugs in various organs may be taken into account in drug design, in order to develop drug molecules which have both desired pharmacological activities and pharmacokinetic properties which would make them useful therapeutic agents. However, detailed structural knowledge of transporter proteins involved in absorption, distribution and elimination is still too limited to be taken directly into account in target-based drug design projects.

6.5 Prodrug Targets

6.5.1 Dipeptide Transporters

Dipeptide transporters (PEPTs) are involved in transport of di- and tripeptides across plasma membranes in the small intestine and kidney proximal tubules. These transporters are important for efficient absorption of protein ingestion products. Dipeptide transporters are H^+-coupled and localized in brush border membranes. They mediate absorption of certain drugs like cephalosporins, β-lactam antibiotics and ACE inhibitors [158]. For example,

whilst methyldopa is poorly absorbed from the intestines, when it is converted to a dipeptyl derivative it is a substrate for dipeptide transporters, and is more efficiently absorbed.

Dipeptide transporters may be exploited for improving intestinal absorption of pharmacologically active amino acids. Therefore, prodrugs targeting the PEPT1 transporter may improve the oral bioavailability of drugs with low intestinal membrane permeability. Structural knowledge of the peptide transporters is important for designing peptidomimetics that may facilitate drug transport across the intestinal epithelium [159–161]. The PEPT transporters belong to the MFS family of transporters, indicating that structural modelling based on the X-ray crystal structure of Lac Permease [70] may provide important structural information about the PEPT transporters.

7 Conclusions

Improved methods in molecular biology, biochemistry, crystallization and X-ray crystallographic data collection and processing have presented the possibility of automation of the different steps of protein expression and structure determination. Together with developments in genomic sequencing, these technical improvements have increased the number of known 3D protein structures. These technical advances have also provided insight into an unprecedented number of potential drug targets and created an environment for the emergence of new strategies for drug discovery. The improvements in technology have also increased the number of available atomic-resolution structures of membrane transporter proteins. However, experimental methods for 3D structure determination of membrane proteins are still difficult and remain a significant challenge for structural biology and new drug discovery. Current knowledge of the 3D structures of membrane proteins, including membrane transporters, is therefore still limited. Almost all known membrane transporter structures have been determined with proteins from bacteria. Several of the bacterial transporters of known 3D structure have mammalian counterparts of therapeutic interest, indicating that molecular modelling approaches may be used to generate 3D models of important drug targets based on structural conservation throughout evolution. Membrane transporters constitute a divergent group of proteins with substrates ranging from ions to relatively large organic molecules, and with large conformational flexibility in order to facilitate substrate transport. This suggests that inhibitors may bind to different conformations of a transporter, and that several conformations of a transporter should be considered in a target-based ligand design approach.

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