Centre for Drug Research
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ABCC2 Transporter and α_2-Adrenoceptors: Identification of Novel Compounds and Their Mode of Action

Gloria Wissel

ACADEMIC DISSERTATION

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Cover: Representation of ABCC2 transporter (left) and α_{2A}-adrenoceptor binding chlorpromazine (right).

Abstract

The main goal of this dissertation is the identification of novel modulators acting on ATP Binding Cassette subfamily C member 2 (ABCC2) transporters and α_2-adrenoceptors subtypes. In order to reach this goal, a combination of experimental and computational approaches are used.

The first protein presented in this dissertation is the ABCC2 transporter, also known as the multidrug resistance associated protein 2 (MRP2). ABCC2 is an efflux transporter expressed in polarized cells where it effluxes a variety of both endogenous and exogenous molecules out of the cell. A common way to study the interactions between small molecules and the ABCC2 transporter is by vesicle transport assays. Commercially available assays use different probes to define the ABCC2 transport. A small set of eight compounds and, subsequently a larger library of compounds were tested with different assays with the intent to identify the effect that small molecules have on the ABCC2 transport. In addition from the larger library, 16 inhibitors have been identified and classification models were built to identify important descriptors, which were able to discriminate inhibitors from inactive molecules. Structure-activity relationships (SAR) of four scaffolds of ABCC2 modulators are also presented. In addition, some unpublished results are presented with further insights the SAR of ABCC2 modulators.

The other proteins included in this dissertation are the three subtypes of the α_2-adrenoceptors. α_2-adrenoceptors are G protein-coupled receptors involved in the signalling pathway of adrenaline and noradrenaline. To date not many subtype selective molecules are present in the market. Subtype selective molecules could be used in treatment of high blood pressure, in the alleviation of withdrawal symptoms, and as anaesthetic with fewer side effects than the current drugs. To define the affinity of a small set of antagonists and outline the involvement of the first transmembrane helix in ligand binding, a competition binding assay has been used with chimera receptors where the first transmembrane helix has been swapped between the three subtypes. Molecular modelling has been used to explain the different binding affinities to the chimera receptors. Additionally, the aim was to identify novel α_{2B}-adrenoceptor selective compounds, thus a mid-sized library has been screened using a miniaturized binding assay. Hierarchical classification and chemoinformatics analysis has been used to visualize and analyse the screening results.

Acknowledgements

My PhD work has been carried out during five long and intensive years at the Centre for Drug Research, University of Helsinki. This thesis is the result of the efforts of many people and I am deeply thankful for all their support!

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When I started in 2010, the Computational Drug Discovery group was made of only three PhD students and now the group has more than doubled. It has been a great group to be part of, we had really fun adventures from Lapland to Tallinn but surely the best and most memorable ones are the annual retreats to sauna and avantouinti. I have learned so much from all of them in these years, scientifically and personally. I would thank the entire group for the patience they showed me especially in the last months and for all the support that they have provided in this long and difficult process in becoming a Doctor. Thank you!

The advantage of being part of the centre for drug research (CDR) is being part of an amazing group of young scientists. Thanks to all the presentation, retreats, coffee and discussion, now I know about eyes, proteins, cells, virus, molecular dynamics and much more. It has been by far more fun than learning all those things from books. It has been a great pleasure to explore and organize events together, making this experience at the University of Helsinki forever memorable.

During these years I was part of two divisions, the Pharmaceutical Chemistry and Technology and the Pharmaceutical Biosciences, this gave me the unique advantage of knowing many people and participating to many different events, I am grateful for all people that I have met in these years!

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I would like to thank my family, for the support they have shown in these years with love and sympathy. Thank you, for supporting my love for science and pushing me to be always the best version of myself.

The last thought goes to Francesco that made me believe that happiness is only real when shared.

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List of original publications

This dissertation is based on the following publications, which will be hereafter referred with Roman numbers. Publications are reprinted with the permission from the publishers.

I. Kidron H*, **Wissel G***, Manevski N, Häkli M, Ketola RA, Finel M, Yliperttula M, Xhaard H, Urtti A. Impact of probe compound in MRP2 vesicular transport assays. Eur. J. Pharm. Sci. 2012, 46:100-105.

II. **Wissel G*,** Kudryavtsev P*, Ghemtio L, Manevski N, Tammela P, Urtti A, Wipf P, Kidron H, Xhaard H. Exploring the structure-activity relationships of ABCC2 modulators using a screening approach. Bioorg. Med. Chem., 2015, *in press.*

III. Laurila J, **Wissel G**, Xhaard H, Ruuskanen J, Johnson M, Scheinin M. Involvement of the first transmembrane segment of human α2-adrenoceptors in the subtype-selective binding of chlorpromazine, spiperone and spiroxatrine. Br. J. Pharmacol. 2011, 164:1558-1572.

IV. Fallarero A, Pohjanoksa K, **Wissel G**, Parkkisenniemi-Kinnunen U-M, Xhaard H, Scheinin M, and Vuorela P. High-throughput screening with a miniaturized radioligand competition assay identifies new modulators of human α_2-adrenoceptors. Eur. J. Pharm. Sci. 2012, 47:941-951.

* With equal contribution

Additional publication not included in the dissertation.

Kiriazis A, Boije af Gennäs G, Talman V, Ekokoski E, Ruotsalainen T, Kylänlahti I, Rüffer T, **Wissel G**, Xhaard H, Lang H, K. Tuominen R, Yli-Kauhaluoma J. Stereoselective synthesis of (3-aminodecahydro-1,4-methanonaphthalen-2-yl) methanols targeted to the C1 domain of protein kinase C. Tetrahedron. 2011, 67:8665-8670.

Personal contribution

I. I performed the assays and collected the data for the study while the data analysis, interpretation, and writing was done together with Dr. Heidi Kidron.

II. I contributed to the majority of the steps of the publication including the preparation of the manuscript, but was not involved in building the QSAR model.

III. I built the homology models, performed the docking studies, and wrote the modelling section in close collaboration with Dr. Henri Xhaard.

IV. I contributed to the chemoinformatics (clustering and classifying the screening results) and docking sections and to the writing of those sections together with Dr. Henri Xhaard.

Abbreviations

ABC	ATP Binding Cassette superfamily
ABCC	ATP Binding Cassette subfamily
ABCC2	ATP Binding Cassette subfamily C member 2, *also known as* canalicular multispecific organic anion transporter 1, and multidrug resistance associated protein 2
ATP	Adenosine-5'-triphosphate
B_{max}	Receptor density
cAMP	Cyclic adenosine monophosphate
CDCF	5 (and 6)-carboxy-2', 7'-dichlorofluorescein
cDNA	complementary DNA
CEC	Chloroethylclonidine
CFTR	Cystic fibrosis transmembrane conductance regulator
CHO	Ovarian cells isolated from Chinese hamster
cMOAT1	*see* ABCC2
DDI	Drug-drug Interaction
EG	β-Estradiol 17-β-D-glucuronide
EDTA	Ethylenediaminetetraacetic acid
EMA	European Medicines Agency
ε	Dielectric constant
FDA	US Food and Drug Administration
GDP	Guanosine-5'- diphosphate
GPCR	G protein-coupled receptor
G-protein	Guanine nucleotide binding protein
GSH	Glutathione
GTP	Guanosine-5'- triphosphate
HTS	High Throughput Screening
IC_{50}	The molar concentration of an unlabeled agonist or antagonist that inhibits the binding of the radioligand by 50%; in the case of transporters: that inhibits the probe transport by 50%.
J_{max}	Maximum velocity of transport
K_a	Association constant
K_d	Dissociation constant
K_i	Inhibition constant
K_m	Concentration of substrate at half V_{max}
K_t	Concentration of substrate at half J_{max}
LTC_4	Leukotriene C4
MDR	Multidrug resistance

mdr1a	Multidrug resistance protein 1A from *Mus musculus*
MsbA	Lipid A export ATP bindin/permease protein from *Salmonella typhimurium*
MRP2	*see* ABCC2
MTSCE	2-aminoethyl methanethiosulfonate
NBD	Nucleotide binding domains
PAINS	Pan Assay Interference Compounds
QSAR	Quantitative structure–activity relationships
RSMD	Root mean square deviation
R_t	Unitary turnover rate for transporters
SAR	Structure–activity relationships
Sav1866	ABC transporter from *Staphylococcus aureus*
Sf9	Ovarian cells isolated from *Spodoptera frugiperda*
SVM	Support vector machines
SUR	Sulfonylurea receptors
TM	Transmembrane
TMD	Transmembrane domains
VT-assay	Vesicular transport assay
V_{max}	Maximum velocity of turnover

1. Introduction

ATP Binding Cassette subfamily C member 2 (ABCC2) and the α_2-adrenoceptors are intrinsic membrane proteins and are essential in the regulation and control of many biochemical functions. The function of membrane proteins can be modulated by endogenous or exogenous compounds. The general aim of this dissertation is the identification of novel compounds and the characterization of their mode of action, which is central in the understanding of the biological role of these proteins. With this intent, screening methodologies, chemoinformatics, and homology modelling approaches are used.

This dissertation focuses on the poorly understood ATP Binding Cassette subfamily C member 2 (ABCC2) transporter, a member of the ATP Binding Cassette family (ABC). ABC transporters control the movement of endogenous/exogenous molecules across membranes, and can have an important role in defining drug pharmacokinetics. ABCC2 is expressed at important pharmacological barriers. It is localised, for example, in the basolateral membranes of hepatocytes where it has a critical role in the biliary elimination of conjugated metabolites. In addition, it has been suggested that ABCC2 is responsible for the increase of multidrug resistance in cancer cells, promoting the efflux of chemotherapeutic agents. Thus, inhibitors of ABCC2 might be used to overcome multidrug resistance. Early predictions of ABCC2 interaction with investigational drugs would be beneficial to predict drug pharmacokinetics and the possibility of drug-drug interactions.

The ABCC2 project results in two peer-review publication (referred as Publication I and Publication II). In both publication the identification and discussion of probe-dependent modulators is presented. In Publication II, screening results identified novel low µM inhibitors and predictive models were built able to discriminate inhibitors from inactive molecules. Further characterization of the structure-activity relationship of ABCC2 inhibitors is presented in the Additional Unpublished results.

α_2-adrenoceptors are G protein-coupled receptors, humans and other mammalian species have three α_2-adrenoceptors subtypes that share a high structural similarity, especially in the transmembrane regions. The overall focus of the α_2-adrenoceptor studies is the design/discovery of low molecular weight molecules able to discriminate α_2-adrenoceptor from other G protein-coupled receptors as well as among the three human subtypes (i.e. subtype-selective molecules), to be used as therapeutic molecules with low side effects. Current clinically available α_2-adrenoceptors drugs have only marginal subtype specificity, which limits the therapeutic usefulness due to side

effects. Selective molecule could be used in treating high blood pressure, in the alleviation of withdrawal symptoms, and as anaesthetic with fewer side effects than the current drugs.

Two studies on α_2-adrenoceptor subtypes are presented in this dissertation (Publication III and IV). The experimental part of these studies was conducted by the collaborators before my involvement in the projects. Consequently, my part in this work is purely retrospective, ie. data analysis. The focus of Publication III was to define the involvement of first transmembrane helix in the binding of a series of antagonist. In Publication IV, the aim was to identify new α_2-adrenoceptors subtype selective ligands.

2. Review of literature

2.1.Membrane proteins

Cell membranes are heterogeneous assemblies of lipids, proteins and carbohydrates that form an approximately 35Å thick layer. Phospholipids, sphingolipids, and sterols (like cholesterol) spontaneously organize themselves in a bilayer where the polar heads are opposite to each other. The composition of the cell membrane additionally, differs between the inner leaflet and the outer leaflet. For example, carbohydrates are involved in cell recognition and are normally linked to proteins or lipids only in the outer leaflet of the membrane. Membrane proteins can be either interacting only with the surface of the membrane (peripheral membrane proteins) or they can be embedded in the bilayer (intrinsic membrane proteins).

Intrinsic membrane proteins mainly have non-polar amino acids pointing towards the bilayer, making hydrophobic interactions. The core of the cell membrane is composed of the hydrophobic lipid tails and it is approximately 20Å thick, suggesting the need of 20 amino acids to cross it. To minimize the exposure to the membrane hydrophobic core the peptide main chain forms hydrogen bonds and predominantly arranges in α-helices (White & Wimley 1999).

Membrane proteins have an essential role in the regulation and control of many biochemical pathways and can be classified based on their function as cell surface proteins, cell adhesion proteins, cytoskeleton attachment proteins, enzymes, channels, transporters, or receptors.

The ATP Binding Cassette subfamily C member 2 (ABCC2) is a transporter, member of the ATP Binding Cassette (ABC) family. Transporters are specialized proteins that help the translocation of molecules across the cell membranes. Passive transport, occurs when the translocation does not directly require chemical energy, as the transported molecules follow their concentration gradient. Active transport instead requires energy to transport molecules across the bilayer; in the case of ABC transporters the energy source is the hydrolysis of ATP. Additionally, transporters can be divided into importers and exporters, depending on the directionality of the transport. The largest family of human efflux transporters is the ATP binding cassette family (ABC) (Dean et al. 2001a). More than 400 transporters have been identified in the human genome that are likely to be associated with pharmacokinetics and safety

profiles for drugs (Giacomini et al. 2010). Genetic variants of these membrane transporters are known to cause serious metabolic disorders (e.g. cystic fibrosis) (Gottesman & Ambudkar 2001)

α_2-adrenoceptors are G protein-coupled receptors (GPCR) that bind adrenaline and noradrenaline. GPCRs are intrinsic membrane receptors that recognise different extracellular signals such as the variation in the concentration of ions, glucose, oxygen, or light and convert it into an intracellular signal. It has been proposed that more than 60% of current drug targets are membrane proteins located at the cell-surface, with the GPCR as the largest family (Overington et al. 2006). Many polymorphisms of GPCRs have been identified, showing mutations in coding and no-coding variants (Rana et al. 2001). Mutant GPCR genes and proteins are associated with several clinical conditions, reviewed in Schöneberg et al. 2004. For example, mutation on the gene that codes for the arginine vasopressin receptor 2 (AVPR2) has shown to cause nephorogenic diabetes insipidus (Knoers 1993). Additionally, mutations can alter the binding site of the receptor, modify its signalling, alter the expression levels, or even modify the ratio between the inactive and the active population of receptors (Thompson et al. 2005; Spiegel 1996; Zalewska et al. 2014).

2.1.1. Challenges in the study of membrane proteins

Functional and biochemical studies on membrane proteins are challenging due to relatively hydrophobic surface and unstable nature of membrane proteins. For the most part, membrane proteins are not expressed in high concentration in native cell membranes; therefore, overexpression is needed for functional and structural studies. The optimization of the overexpression process is crucial. Many different expression systems are used that differ in the post-translational modifications, protein yield, and stability. The different expression systems, solubilisation, and purification methods for membrane proteins have been discussed in Junge et al. 2008 and will not be discussed further here.

Proteins have to be correctly folded to be functional and membrane proteins can fold correctly only if targeted into the cell membrane. This process is controlled by specific machinery (translocon) that is encoded by a characteristic sequence of amino acids (von Heijne 2006). In addition, the function of the protein is influenced by the composition of the membrane, since there are many interactions between the lipids and the embedded protein. Many techniques do not allow the study and analyse of proteins in such lipophilic and diverse environment, thus proteins have to be extracted and studied in detergent or specific lipid settings (Seddon et al. 2004).

2.1.2. Prediction of membrane protein structure

While in the last years major developments have been made to improve membrane protein crystallization, only less than 3% of crystallized proteins are membrane proteins (Berman et al. 2000). Therefore, several predictive tools for membrane protein structure have been developed.

Protein structure prediction has developed intensively beginning in 1970's (Frishman 2010). The simplest methods predict the amino acids that belong to transmembrane segments utilizing hydropathy plots and hydrophobic moment plots (Kyte & Doolittle 1982). More modern methods have been implemented that use, for example, machine learning algorithms and hidden Markov models (e.g. transmembrane hidden Markov model TMHMM) (Tusnády & Simon 2001). Additionally, it is possible to predict the orientation of the protein identifying the intracellular and extracellular regions, using the positive inside rule. The positive inside rule states that a net positive balance, due to positively charged amino acids, is found predominantly intracellularly (von Heijne & Gavel 1988).

In addition to the hydrophobicity, it has been shown by multiple alignments that the inner core of the protein is more conserved than the periphery. Such information has proven to be a very useful tool to indicate the protein interior (Samatey et al. 1995). In addition, new programs have emerged that incorporate experimental results such as the known location of N-terminus or C-terminus, with a further increase of 10% the prediction accuracy (Tusnády & Simon 2001). Prediction of membrane protein topology based on the amino acid sequence is reviewed by Casadio et al. 2003.

2.2. Protein-ligand binding

2.2.1. Protein-ligand equilibrium

The interaction between a ligand and a protein can be represented as follows (Eq. 1), where k_1 is the association rate constant and k_{-1} is the dissociation rate constant.

$$\text{Protein (P)} + \text{Ligand (L)} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Protein-Ligand (PL)} \qquad \text{Eq. 1}$$

At equilibrium, following the law of mass action, the affinity between a protein and its ligand can be represented by the dissociation constant K_d and its reciprocal is the association constant K_a (Eq. 2).

$$K_d = \frac{k_{-1}}{k_1} = \frac{[P][L]}{[PL]} = \frac{1}{K_a} \qquad \text{Eq. 2}$$

The total number of binding sites (B_{max}) is given by the sum of all occupied and unoccupied sites (Eq. 3).

$$B_{max} = P + PL \qquad \text{Eq. 3}$$

Then, K_d can be rewritten (Eq. 4).

$$K_d = L\,(B_{max} - PL) / PL \qquad \text{Eq. 4}$$

2.2.2. Gibbs free energy

At equilibrium, the change in free energy of the system is represented as the change in Gibbs free energy (ΔG) and expressed as KJ/mol. ΔG is directly proportional to the affinity between the protein and the ligand (K_d), the temperature (T), and the ideal gas constant (R). Additionally, the energy of binding can be represented as the change in enthalpy (ΔH) and the change in entropy (ΔS) at a certain temperature (Eq. 5).

$$\Delta G = RT \ln K_d = -RT \ln K_a = \Delta H - T\,\Delta S \qquad \text{Eq. 5}$$

A spontaneous reaction occurs if the energy of the system decreases, thus having a negative ΔG. The enthalpy parameter represents approximately the strength and specificity of the molecular interaction between ligand and protein that will be further discussed in 2.2.4. Entropy, instead, represents the disorder of the system; this parameter is connected to the loss of translational and rotational degrees of freedom of both partners (protein as well as ligand). Desolvation and solvent-reorganization contribute to both the enthalpy and entropy of binding.

2.2.3. Theories of ligand binding

The first theory about ligand binding, *the lock and key theory*, was proposed in 1894 by the German chemist, Emil Fischer. An updated model presented in 1958 by biochemist Daniel E. Koshland, Jr. suggested the *induced fit theory*, where both ligand and protein adapt to each other when interacting. This model suggests that the binding interaction is not static but dynamic process, where both ligand and protein rearrange to interact with each other.

These models are based on the existence of a primary site, the orthosteric site, which binds the endogenous ligand, and after binding, produces a biological effect. Besides the orthosteric site, molecules can binding to a topographically distinct sites called allosteric sites. Molecules that bind to the allosteric site(s) can enhance or inhibit the binding of the endogenous ligand to the orthosteric site (in the case of an enzyme, the catalytic activity also can be affected). Allostery is mediated through conformational changes that happen within a protein (from one site to another) or can be transferred to the neighbouring protein when it occurs in oligomeric protein complexes (Crick & Wyman 2013; Monod et al. 1965).

GPCRs are naturally allosteric proteins as they possess more than one binding site topographically separated (Bouvier 2001). The G-protein (coupled to the receptor) is in fact the best-known allosteric modulator of GPCR agonist binding (Christopoulos & Kenakin 2002; May et al. 2010). In addition, protein-protein interaction between GPCRs (homo- and hetero-dimers) and a variety of other proteins, confirm the allosteric nature of GPCRs (reviewed in Brady & Limbird 2002).

2.2.4. Molecular interactions

The most common interaction between ligands and proteins are covalent, electrostatic, Van der Waals, hydrogen bond, and π-interactions.

❖ *Covalent*

Covalent interactions, which involve the share of electrons, are the most stable chemical interaction between two atoms. Covalent interaction are associated with safety and toxicity concerns due to the long duration of action (Mah et al. 2014). When an inhibitor is covalently bound to an enzyme, the duration of the interaction maybe be so long that it may be impossible to reverse, for example in case of overdose. Of the marketed drugs that act on enzymes, about 30% of them act through covalent interaction (Robertson 2005).

❖ *Electrostatic*

Electrostatic interactions occur between two atoms that have an electrostatic charge (cation or anion) and can be of attractive or repulsive nature. An attractive force, between two atoms with opposite net charge (negative/positive), is considered here (Eq. 6). The interacting force (F) depends on the charges (q_1, q_2), the square of the distance (r^2), and the dielectric constant (K_ε) that can change depending on the environment. The dielectric constant is about ~80 in water and usually lower in proteins (about four inside a receptor's hydrophobic binding pocket) (Rubinstein & Sherman 2004).

$$F = (K_\varepsilon)\frac{(q_1 * q_2)}{r^2} \qquad \text{Eq. 6}$$

❖ *Van der Waals*

Van der Waals interactions are a rather weak type of interaction that occur between dipoles and induced dipoles. A dipole is by definition a partial charge that is not uniformly distributed over the molecule. Dipoles can be permanent or transient in time: instant or induced. Instant dipoles occur when electrons are temporarily concentrated on one part of the molecule. A molecule with a permanent dipole or charge can affect another molecules' electron cloud and induce a dipole moment. Examples of the most common Van der Waals interactions are presented in Figure 1.

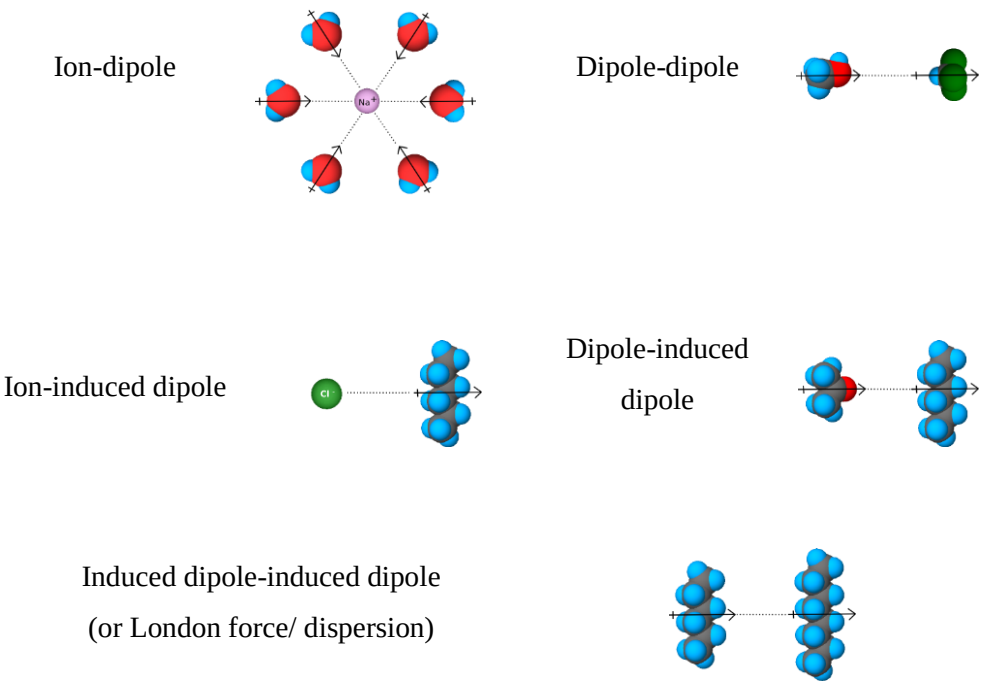


Figure 1 The most common Van der Waals interactions. In red oxygen atoms, in light blue hydrogens, in green chlorine atoms and in grey carbons.

❖ *Hydrogen bonds*

Hydrogen bonds are weak interactions that are grouped independently since they cannot be explained by Van der Waals interactions, as they have a partial covalent component. Hydrogen bonds take place between an electronegative atom and a hydrogen atom covalently bound to a second electronegative atom. Intermolecular hydrogen bonds are responsible for the high boiling point of water when compared to other small molecule hydrocarbons. Water molecules can be both hydrogen bond acceptors and hydrogen bond donors. A water molecule acts as a hydrogen bond acceptor when its oxygen acts as the electronegative counterpart for the interaction and acts as a hydrogen bond donor when the hydrogen is involved in the bond.

❖ *π-interactions*

Ligand-protein interaction can be additionally stabilized with interactions that involve π-systems, the most common ones are cation-π, π-π and C/N/OH-π that are presented in Figure 2. π-systems are conjugated systems that occur when p-orbitals (p molecular orbitals) overlap and π-electrons can be delocalised in the conjugated system. In the case of aromatic rings, it creates an electron-rich system over and under the aromatic ring and an electron-poor region at the level planar to the ring.

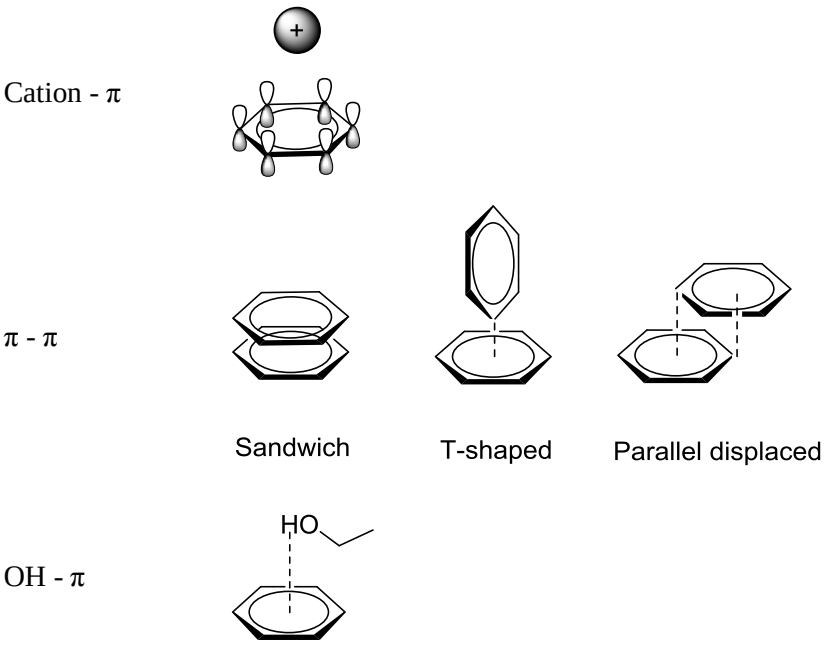
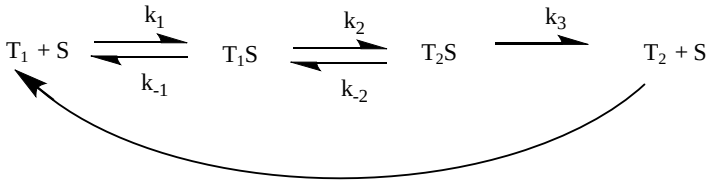


Figure 2 An example of π-interactions.

2.2.5. Transport kinetics

Transport kinetics can be described by analogy to the enzyme kinetic models initially proposed by Leonor Michaelis and Maud Menten in 1913 (original paper has been translated in English by Johnson & Goody 2011). The main difference between enzymes and transporters is that enzymes break and form new bonds in substrate molecules, while transporters translocate their substrate(s) across the membrane. Formally the efflux transport can be described as following (Eq. 7), with T_1 representing the inward facing conformation and T_2 the outward facing conformation. In the case of active transport, the transport cycle (the conversion between T_1 and T_2

and vice versa) is energy dependent. Specifics of ABC transporters are presented in section 2.3.1 in the Alternative access model paragraph.



Eq. 7

The first assumption of the Michaelis-Menten enzyme kinetic model is that at the steady state, the concentration of the enzyme-substrate complex is constant over time and independent of the concentration of the substrate. This happens only when the concentration of substrate is so high that all the enzymes are saturated, thus a further increase of substrate will not change the rate of catalysis. In this condition of high substrate concentration, it is possible to identify a plateau, where the rate of reaction is constant, described as V_{max} (Figure 3). The second assumption is that the reaction proceeds only to one product; hence, the equilibrium is shifted to the right. When all these assumptions are in place, it is possible to plot the variation of the reaction rate against the variation of the substrate concentration (Figure 3) and calculate the reaction rate with the Michaelis-Menten equation (Eq. 8). Where K_m is the concentration of the substrate when the rate of reaction is half of the maximal rate, V_{max}, the K_m parameter is used to compare the binding affinity of different substrates; a lower value of K_m indicates that a lower concentration of substrate is needed to reach half of the maximum rate.

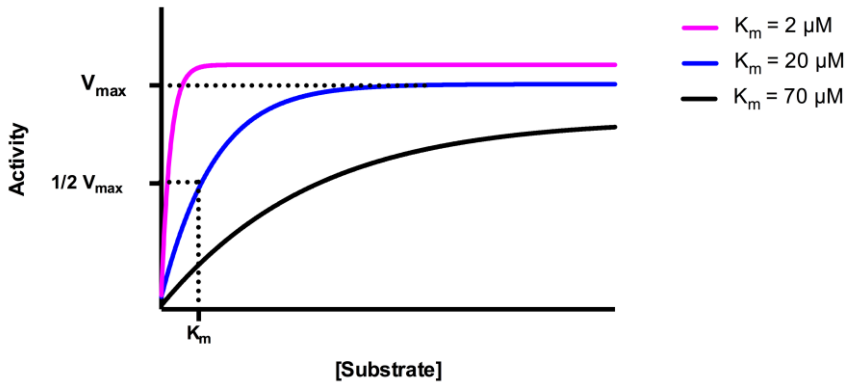


Figure 3 Michaelis-Menten kinetics. Variation of the reaction rate is plotted against the increase of substrate concentration. V_{max} is the maximum rate of enzyme catalysis; K_m concentration of the substrate at half of V_{max}.

$$\text{Activity} = \frac{V_{max} * \text{S}}{K_m + S} \qquad \text{Eq. 8}$$

Similar to enzymes, transporters are proposed to have a main substrate binding site that can be saturated and inhibited and, therefore, the mechanism of transport can be represented with Michalis-Menten kinetics (Bentz et al. 2005).

At the steady state (Eq. 7), the transporter substrate complexes (T_{1S} and T_{2S}) are considered equivalent. The rate of transport (J) is measured as the function of the translocation of the substrate. Similar to enzymes, at high concentration, the rate capacity approaches J_{max}; thus, the rate of the transport can then be calculated with the Michaelis-Menten equation (Eq. 9). As with enzymes, K_t is the concentration of the substrate at half of the maximal transport rate.

$$\text{J} = \frac{J_{max} * \text{S}}{K_t + S} \qquad \text{Eq. 9}$$

Additionally, for transporters it is possible to calculate the unitary turnover rate (R_t) that is defined as the number of molecules transported across the membrane in the unity of time. It represents how fast the transporter cycles occur, normally expressed as cycles per second and is calculated (in Eq. 10) with B_{max} as the total amount of transporters.

$$R_t = \frac{J_{max}}{B_{max}} \qquad \text{Eq. 10}$$

In the case of transporters it is important to define two groups of interacting ligands, transported molecules (substrates) or non-transported ligands.

2.2.6. Two-state receptor theory

Receptors are specialized proteins that convert extracellular information into an intracellular signal. The classic two state receptor theory proposed in 1965 describes the interaction between ligands and receptors on a molecular level. Ligand binding to the receptor changes the conformation of the receptor from the inactive (R) to the active (R*) conformation (Monod et al. 1965). This assumes that equilibrium between the active and the inactive conformation exists (Eq. 11). In addition, receptors may signal in the absence of a ligand, suggesting a spontaneous conversion between R and R*.

$$\text{R} \rightleftharpoons \text{R}^* \qquad \text{Eq. 11}$$

When ligands bind, two are the possibilities that may coexist. The conformational selection theory proposes the ligand to stabilize one or the other conformation, shifting the equilibrium between the two forms. The conformational induction theory instead suggests that the ligand actively promotes the conformational change between the two conformations. At the moment, it is not possible to validate or invalidate one or the other theory experimentally. In addition, it has been proposed that not only receptors can interconvert between the two states R and R* but can include some intermediate transitions stages (Park et al. 2008).

The propensity of the drug to bind to the receptor is called affinity, it is normally calculated as IC_{50} or K_i; while, the extent of the functional changes imparted by the receptor are called efficacy.

Full agonists are ligands that, after all the receptors are occupied, can promote the maximal response to be reached (full efficacy is reached). Partial agonists, instead are ligands that produce less than the full effect even at saturation; thus acting like an antagonist in the presence of a full agonist (blocking the full effect).

An inverse agonist, instead, imparts the opposite effect than the agonist, stabilizing the inactive conformation of the receptors (R), thus shifting the equilibrium to the left (Eq. 11). This shift of the equilibrium can lower the baseline activity to null or, in some cases, even cause inverse activity of the receptor. Neutral antagonist are by definition ligands able to bind to both R and R*, preventing any agonist response but not altering the equilibrium between R and R* and not changing the baseline activity (Figure 4). After the identification of constitutively active receptors, many ligands initially identified as antagonist, with a negative efficacies, have been later reclassified as inverse agonist (Gilchrist 2007).

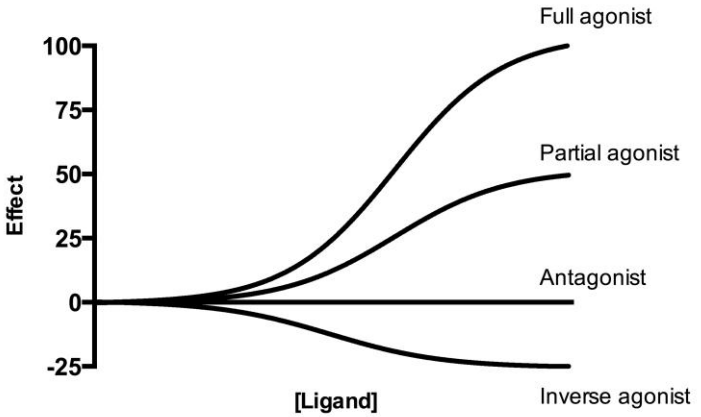


Figure 4 Dose response curves. The variation of the observed activity of the receptor plotted against the increase of the concentration of different ligands. The increase of the concentration of the agonist will fully activate the receptor (reaching full response at saturation). Partial agonists instead, even at saturation, are not able to fully activate the receptor. Antagonists do not alter the base line activity of the receptor (neither increasing nor decreasing). An inverse agonist at saturation can block fully the receptor activity, like in this case eliminating even the base line activity.

For GPCRs, it has been recognized that many may be constitutively active, able to signal in the absence of a bonded ligand confirmed in endogenous systems (Tiberi & Caron 1994). In addition, a tertiary model can be proposed where the receptor is in equilibrium with the G-protein and the cubic ternary model that includes an allosteric modulator (Figure 5).

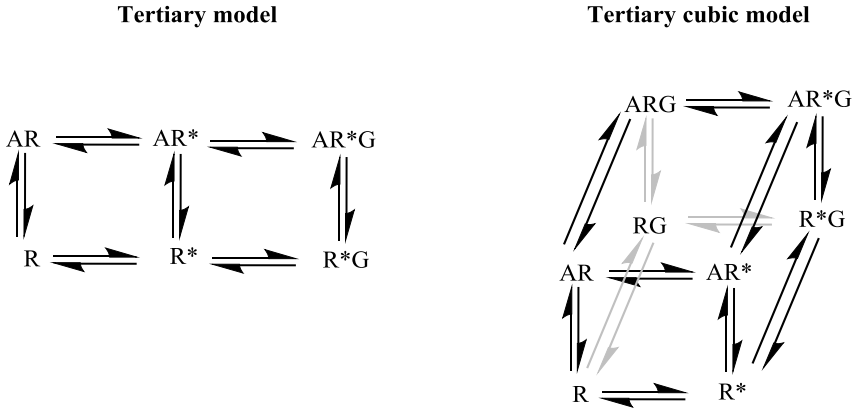


Figure 5 Tertiary model and cubic tertiary model. On the left the tertiary model and on the right the cubic tertiary model. Agonist (A), receptor (R), G-protein (G) and activated conformation (*).

2.3.ABC transporters

2.3.1. ATP binding cassette family

The ATP binding cassette (ABC) systems are one of the most ancient protein families, representatives of such family can be found in both prokaryotic and eukaryotic cells (reviewed in Rees et al. 2009). ABC transporters are able to carry across the membrane a diverse range of molecules, from small ionic compounds to very hydrophobic molecules. Functional transporters consist of two transmembrane domains (TMD) and two nucleotide binding domains (NBD). Some transporters are expressed as fully functional proteins containing two NBD and two TMD, e.g ABCB1; or as half transporters with one NBD and one TMD that need to dimerize to be fully functional e.g. ABCG2

The transmembrane domains (TMDs), which are less conserved than nucleotide binding domains (NBD), are responsible for binding and translocation of the substrates across the membrane. These TMDs are not present in all human members of the family and some members of the ABC family are most probably not transporters (Dean et al. 2001a).

The nucleotide binding domain (NBD) is a highly conserved domain that binds and creates the catalytic site for the ATP hydrolysis. Several conserved motifs can be identified: the walker A (GXXGXGKS/T), the walker B (ΦΦΦΦD, Φ is a hydrophobic residue), and the signature motive C (LSGGQ) that are specific for the ABC transporters (Schmitt et al. 2003). Additionally, three conserved loops that are important for the catalytic function can be identified: the A loop (an aromatic loop normally containing at least one tyrosine), the D loop (with a conserved SALD motif), the Q loop (composed of about eight amino acids including a conserved asparagine), and the H loop or switch region (present in the C-terminal part of the domain with a conserved histidine) (ter Beek et al. 2014).

❖ *Classification*

In humans, 48 ABC efflux transporters have been classified based on the phylogenetic analysis of NBD into seven families (A-G) (Dean et al. 2001a).

The ABCA subfamily is composed of 12 proteins that regulate the homeostasis of cholesterol and lipids (Kaminski et al. 2006). It has been demonstrated that the

mutation on ABCA1 can cause the Tangier disease characterized by the accumulation of cholesterol in many tissues (Brooks-Wilson et al. 1999).

The second subfamily, the ABCB it is even called the multidrug resistant (MDR) family as many members of this family cause multidrug resistance. This subfamily is composed of four full transporters and seven half transporters. The ABCB subfamily is known to transport a wide range of generally hydrophobic molecules (Dean et al. 2001b). The best characterised transporter of the subfamily is ABCB1 commonly known as MDR1 or P-gp (Palmeira et al. 2012).

The third subfamily, ABCC, is also known as the multidrug resistant associated proteins (MRP) family as nine of its members confer multidrug resistance. The cystic fibrosis gene (CFTR, ABCC7) is an important transporter in the subfamily; inborn mutations of these gene have shown to cause cystic fibrosis (Gottesman & Ambudkar 2001).

The ABCD subfamily is composed of four members encoded as half transporters functioning as homo- or hetero-dimer, and are known to transport very long fatty acids-CoA (Kemp et al. 2011). Mutations of the ABCD1 are linked X-linked adrenoleukodystrophy (Mosser et al. 1993).

The ABCE subfamily is composed of a single protein (also known as ribonuclease-L inhibitor) expressed as a single NBD, without any TMD, thus unlikely to function as a transporter (Karcher et al. 2008). ABCE is suggested to promote interferon activity (Bisbal et al. 1995).

Three proteins are grouped in the ABCF subfamily, as well as the ABCE are expressed without a TMD, thus not functioning as transporter. It has been proposed that they could be involved in ribosome biogenesis or protein synthesis (Dong et al. 2005; Tyzack et al. 2000).

The white or ABCG subfamily of ABC transports is composed of five members encoded as reverse half transporters and are known to transport lipids and sterols (Wang et al. 2013). ABCG1 and ABCG4 mediate the transport of cholesterol and high density lipoproteins (Wang et al. 2004).While the most characterized protein in this family is the BCRP or ABCG2 known also as breast cancer resistant protein (BCRP) shown to be expressed in choriocarcinoma cell lines (Bailey-Dell et al. 2001).

❖ *Transporters and pharmacokinetics*

ABC transporters have a central role in secretory epithelia to excrete endogenous metabolites, for example bile salts or bilirubine glucuronides. An important consequence of the presence of efflux transporters in healthy tissues is their impact on pharmacokinetics, i.e. the absorption, distribution and elimination, of many drugs (Cascorbi 2006). For instance, the bioavailability of orally administered substrate drugs is regulated by efflux transporters expressed in intestinal epithelial cells. Additionally, at the blood-brain barrier efflux transporters impede the penetration of drugs into the brain, thereby decreasing the efficacy of treatment directed at the central nervous system, inhibitors in this case could improve drug therapy (Schinkel et al. 1996).

Efflux transporters have been found to be important mediators of drug-drug interactions that may lead to serious adverse reactions. Drug-drug interactions (DDI) are caused by a drug molecule that induces or inhibits a metabolic enzyme, or in this case, a transporter thereby influencing the interactions of the protein with another drug. DDIs can promote or decrease the metabolism or transport of the victim drug and can cause drug induced toxicity or alter the efficacy of drug treatment (Keogh 2012; El-Sheikh 2007). For example, inhibition of efflux transporters in the intestine can lead to several-fold increase in the bioavailability of a victim drug. The bioavailability of irinotecan is increased up to a five-fold when an efflux transporter inhibitor, verapamil, is co-administered (Bansal et al. 2009).

❖ *Multidrug Resistance*

Multidrug resistance (MDR) is a general phenotype in which a human tumour becomes resistant to multiple chemotherapeutic drugs. Drug resistance is one of the main reasons of failures in cancer chemotherapy. The overexpression of ABC transporters in cancer cells can limit the accumulation of the chemotherapeutic drug in the cells, thus causing the cells to become resistant to the drug.

For instance, a clear association of high MDR1 expression in leukemic cells and poor outcome has been demonstrated and therefore inhibitors of MDR1 have been evaluated in clinical trials for chemotherapeutic treatment of acute myeloid leukemia. Unfortunately, the MDR1 inhibitors have not been able to improve the therapeutic outcome (Shaffer et al. 2012).

❖ *Aternative access model with a twist*

The *alternate access* mechanism of transport was initially proposed in 1966 by Jardetzky for membrane pumps, suggesting the presence of a central binding cavity (orthosteric site) that is never simultaneously open to both sides of the membrane (Jardetzky 1966). This suggests that the transporter switches from an inward to an outward conformation.

The presence of these two conformations, suggested by Jardetzky, is in agreement with the solved 3D structures of the ABC transporters. The first full length ABC exporter (Sav1866) was crystallized in 2006 in the outward open conformation with ADP or ATP analogues (AMP-PNP) (Dawson & Locher 2006, 2007). The first nucleotide-free inward open conformation of mouse mdr1 was solved three years later by Aller et al. 2009 and later refined (Li et al. 2014). Additional insight in the mechanism of transport followed, after the low resolution crystal structure of the Lipid A ATP-binding/permease protein (MsbA) lipid flippase from *Salmonella typhimurium* (Ward et al. 2007). Here the "alternative access model with a twist" was proposed, in which the conformational changes to propagate from the NBDs and involve a twist of about 30° of the helices in the TMDs (Ward et al. 2007). The first high resolution (2.9Å) heterodimeric protein (TM287 and TM288) from *Termogota maritma* showed an inward facing conformation with only partially separated NBDs (inward-closed) (Hohl et al. 2012). Recently, an additional intermediate conformation was crystallized, filling the gap between the different conformations and increasing our current knowledge of the mechanism of transport for ABC exporters (Choudhury et al. 2014).

It has been proposed that two ATP molecules are needed for the translocation of the substrate (Senior et al. 1995). Two alternative models have been suggested to define the energy stroke that promotes the drug from the high affinity site (T_1) to the low affinity site (T_2) (Eq. 7, section 2.2.5). The first model proposes that the substrate and the ATP initially bind to the transporter simultaneously and a first hydrolysis is needed for the efflux and the second one to restore the ground state (Sauna & Ambudkar 2000). The second model, postulates that the formation of the NBD dimer is a result of the conformational changes occurring after substrate binding and the two consequent hydrolysis events are needed to restore the transporter in the initial state (Higgins & Linton 2004). Additional studies are needed as it is not possible to validate or invalidate one or the other model experimentally.

Briefly, combining the information obtained from the crystal structures it is possible to propose the efflux transport cycle (Choudhury et al. 2014). Starting from an inward-open conformation, where substrate binding occurs, conformation changes occur so that the transporter rearranges to an inward-closed conformation. Consequently, additional rearrangements bring the transporter to an outward-open conformation where the substrate can be released. After the release the outward conformation closes, outward-occluded, and additional conformation changes restore the transporter to the initial inward conformation (Figure 6).

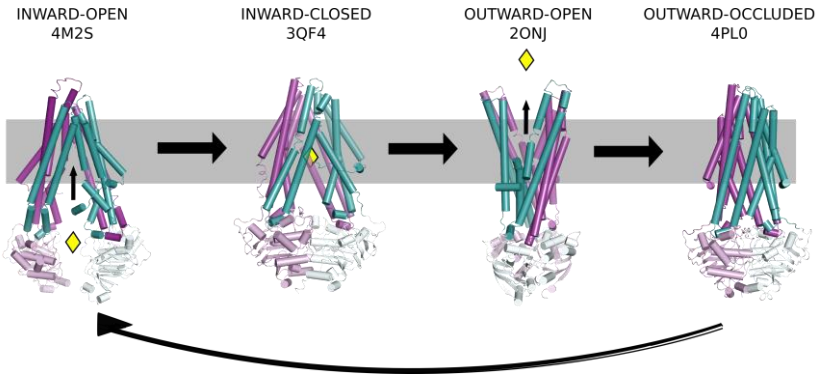


Figure 6 The proposed ABC efflux transport mechanism. This proposed mechanism is explained using the different high resolution ABC efflux crystal structures, crystalized in different conformations. Conformation and pdb codes are in bold. Substrate is represented as yellow rhombus; TMD1 and NBD1 in magenta and light pink respectively while TMD2 and NDB2 in teal and light teal. 4M2S (Aller et al. 2009; Li et al. 2014); 3QF4 (Hohl et al. 2012), 2ONJ (Dawson & Locher 2006; Dawson & Locher 2007) and 4PL0 (Choudhury et al. 2014). The arrows represent the steps in the direction of the transport process. The Figure is adapted from Choudhury et al. 2014.

ABC efflux transporters' binding sites are still weakly characterized, as no crystal structure with a transported substrate is available. Most likely the binding cavity is located in the interface of the two TMDs as shown in the refined crystal structure of mouse mdr1, crystallized with two inhibitors (QZR9-R RR or two QZR9-SSS), and showing a partial overlap of the binding site (Aller et al. 2009; Li et al. 2014).

Pharmacological evidence suggests the presence of two substrates binding site in MDR1, the hoechst and the rhodamine site (Parveen et al. 2011; Shapiro & Ling 1997).

2.3.2. *Multidrug resistance associated protein 2*

ABCC2 or multidrug resistance associated protein 2 (MRP2) is classified into the ABCC family (Dean et al. 2001a). The ABCC family is composed of twelve full transporters: nine of which are multidrug resistance associated proteins (MRP), two sulfonylurea receptors (SUR), and the cystic fibrosis transmembrane conductance regulator (CFTR).

❖ *Stucture*

ABCC2 is a 1545 amino acid protein arranged in 17 transmembrane helices and two homologous intracellular NBDs that bind ATP. The 17 transmembrane helices can be grouped into three transmembrane domains, two homologous domains that constitute the translocation pathway (TMD1 and TMD2), and TMD0, which function is still for a large part unknown (Figure 7).

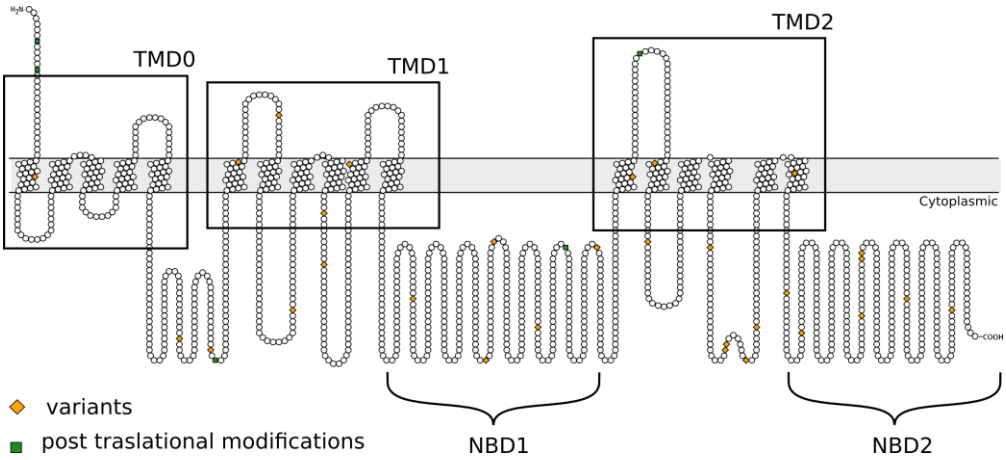


Figure 7 The ABCC2 topology. ABCC2 consists of 1545 amino acids that are organized in three TMDs and two NBDs. Two glycosylation sites are present in the N-terminus and one in the TMD2. The figure was generated using Protter (Omasits et al. 2014) and then modified.

The N-terminal part and TMD0 are proposed to be involved in the correct apical localisation of the transporter in the cell membrane and not important for the translocation of a few tested substrates (Bakos et al. 1996). Swaps between the TMD0s of ABCC2 and ABCC1, a homologous protein that is localized at the basolateral side of polarized membranes, showed that ABCC2-TMD0 is responsible for the apical localisation of the protein and/or stabilizes it into the membrane (Mateus et al. 2002; Konno et al. 2003). Glycosylation at the N-terminus, at amino acids 7 and 12, has also

been suggested to be important in targeting the protein to the apical membrane (Mateus Fernández et al. 2002).

Similarly to MDR1, the pharmacological characterization of ABCC2 showed two different binding sites. Initially it was proposed that ABCC2 could have two drug binding sites, one with high affinity to glutathione (GSH) (G-site) and one with low affinity to GSH and high for drugs (D-site) (Evers et al. 2000). This model have been later revised in a substrate binding site (S-site) and a modulator site (M-site), suggesting that compounds binding to the M-site are not transported but affect the transport of the compounds situated in S-site simulates (Zelcer et al. 2003). Inhibitors of the efflux transporters can thus either compete for binding with the substrate or bind to a separate modulator site.

❖ *Function*

ABC transporters have an important role in drug absorption, distribution, elimination, and drug safety. ABCC2 is expressed in several organs (liver, kidney, and placenta) at the apical side of polarized cells. Initially, ABCC2 has been named the canalicular multi-specific organic anion transporter 1 (cMOAT1) due to its expression at the canalicular membranes of hepatocytes.

Several functional polymorphisms have been identified, but only a small amount of them lead to a non-functional transporter, causing the Dubin-Johnson syndrome. The Dubin-Johnson syndrome is characterized by the accumulation of bilirubin and conjugated bilirubin in the hepatic cells instead of elimination to the bile (Nies & Keppler 2007).

ABCC2 has a broad substrate specificity, transporting across the cell membrane compounds of very diverse structure (Pedersen et al. 2008). Metabolic conjugates are known to be endogenous ABCC2 substrates, these include leukotriene C4, estradiol glucuronide, bilirubine glucuronide, and estrone-3-sulphate (Leier et al. 2000; Paulusma et al. 1999; Cui et al. 1999; Hagmann et al. 1999; Kamisako et al. 1999). In addition, to the endogenous compounds, ABCC2 effluxes exogenous compounds, preventing their toxic accumulation in the cell (reviewed in (van der Schoor et al. 2015). Examples of these molecules are cisplatin, paclitaxel, docetaxel, vinblastine, erythromycin indinavir, ritonavir, and saquinavir (Cui et al. 1999; Huisman et al. 2005; Evers et al. 2000; Agnani et al. 2011; Huisman et al. 2002).

❖ *ABCC2 mediated drug-drug interaction*

Clear evidence of ABCC2 involvement in DDIs has not been presented yet, however, it is plausible that hepatotoxicity may be the result of compounds that inhibit ABCC2 and other transporters of the same family (ABCC3, ABCC4, and ABCC5). At the last international transporter consortium meeting (2013), it has been suggested to investigate ABCC2-mediated interactions if drug-induced hyperbilirubinemia is observed (Hillgren et al. 2013). Currently, regulatory agencies in the US and Europe are advising to study transporter mediated DDI defining the interaction between two ABC transporters (ABCB1 and ABCG) and new investigational drugs (European Medicines Agency 2013; FDA 2012).

Several *in-vitro* systems are used to study the interplay of ABCC2 transporter and drugs, to define drug interaction; the most used ones are based on primary cells lines, recombinant cell line, and plasma vesicles.

Primary cell lines and immortalized cells are used mainly for qualitative studies and to understand mainly the interplay of human transporters (Schrenk et al. 2001). For example, hepatocytes (plated, in suspension or sandwiched) are used to evaluate hepatic uptake and efflux as these cell lines allow the interplay of many transporters. From such systems it is possible to calculate the efflux ratio and intrinsic permeability that have shown to closely relate to the *in-vivo* ones (Polli et al. 2001; Lumen et al. 2010).

Recombinant cell lines, instead, tend to be more robust and reproducible systems and are cultured as polarized monolayers. Oocytes are considered the purest tool to study ABC transporters and are grown in semi permeable supports that allows measurements of the drug in both apical (A>B) and basolateral (B>A) direction. The measurement on both sides of the cell layer is important to understand the impact of passive permeability and can be used to assess the interplay between uptake and efflux (Brouwer et al. 2013). The major pitfall of cell-based systems is the low high throughput and the difficulty in maintenance, thus isolated plasma vesicles are used more.

With plasma vesicles it is possible to study the transport of labelled substrates and the modulation of transported probes. The vesicular transport assay will be further discussed in material and methods paragraph 4.2.1. Additionally, *in-vitro* methods have been presented in detail in Hillgren et al. 2013.

❖ *Multidrug resistance associated protein*

ABCC2 was initially isolated by Taniguki in 1996 from cisplatin resistant cells, thus classifying this transporter as a multidrug resistance associated protein (MRP2) (Taniguchi et al. 1996). It is still not clear if the overexpression of ABCC2 is the cause of multidrug resistance or a mere consequence of the chemotherapy (Borst et al. 1997). Nevertheless, ABCC2 modulation has been investigated to evaluate if the co-administration of ABCC2 inhibitors in chemotherapy is a positive strategy to overcome the multidrug resistance. An example of the use of ABCC2 inhibitors to overcome drug resistance can be the use of montelukas in cancer therapy. Montelukas is an antihistaminic drug that has been identified as a possible/positive adjuvant in combination with taxol and sequinavir (Roy et al. 2009).

2.4. G protein-coupled receptors

2.4.1. General features

G protein-coupled receptors (GPCR) are intrinsic membrane receptors that recognise different extracellular signals and convert it into intracellular signals. In humans, 791 GPCR transcripts have been phylogenetically classified into five different families. The A class or rhodopsine like receptors (662, number of receptor in the class) is the largest family and further divided based on interacting ligand; α (15) binding peptide or amines; β (35) binds peptides; γ (59) binding chemokine, neuropeptides and opioids, *δ* (59) that bind glycoproteins, purine, and olfactory receptors (460) (Fredriksson et al. 2003). Adrenoceptors are classified in the A-α class, as they bind catecholamines (amines).

GPCRs are composed of seven transmembrane helical segments of about 25-35 amino acids, an extracellular N-terminal domain, and an intracellular C-terminal domain. The general fold can be seen from the first mammalian structure, bovine rhodopsin (Palczewski et al. 2000). Each of the seven helices is characterized by conserved amino acids that form the signature of the family (Table 1).

Table 1 Conserved amino acids in the A- α class.

TM1	TM2	TM3	TM4	TM5	TM6	TM7
GxxN	LAxxD	E/DRY/F	W	PxxxxxFxY	FxxxWxP	NP

Specific nomenclatures for GPCR have been developed. The most used is the Ballesteros-Weinstein nomenclature where to the most conserved amino acids in all TM is assigned the helix number and the number of .50 (for example conserved asparagine in TM1 is assigned 1.50) (Ballesteros & Weinstein 1992).

❖ *Signal transduction/ downstream pathways*

Signal transduction occurs intracellularly in response to an extracellular signal. A ligand binding to a receptor causes a conformational change that activates the signalling pathway. In GPCRs, the signalling pathway occurs via GTP proteins (G protein) that is coupled with the receptor intracellularly. In the inactive form the G protein is a trimer consisting of three subunits α, β, and γ, with the G_α subunit binding GDP. After ligand binding, conformational changes of the receptor cause the

dissociation of the trimer into G_{α} (with the exchange of GDP to GTP) and $G_{\beta\gamma}$ that may or may not have been pre-coupled. More than 10 homologues of each of G_{α}, G_{β}, and G_{γ} subunits exist, forming various combinations (Clapham et al. 1995).

The signalling cascade is initiated when the active G_{α} dissociates from the $G_{\beta\gamma}$ dimer and binds with e.g adenylate cyclase, which then leads to the production of a secondary messenger like cAMP. Another common secondary messenger is phosphatidylinositol-2,4-biphosphate and diglycerol that are produced after the activation of phospholipase C. The secondary messenger in turn activates the downstream pathway usually related to gene expression, often through the activation of a kinase (Pearson et al. 2001).

Desensitization is an important process that diminishes the receptor response after its exposure to a ligand (Katz & Thesleff 1957). The short term desensitization occurs via phosphorylation of the intracellular domain by a protein kinase. Phosphorylated GPCR bind the β-arrestin, a cytosolic protein, that mediates the internalization of GPCRs and desensitization, restoring the G protein complex (Tian et al. 2014). The long-term desensitization, instead, occurs through changes in the expression of the receptor and other proteins in the pathway (down regulation).

Additionally, it has been identified that some ligands trigger the β arrestin pathway, preferably to the G protein coupled one (Lohse et al. 1990). In addition, many GPCRs can couple together as hetero- and homo-dimers, which increases the complexity in G protein-coupling even further (Waldhoer et al. 2005).

❖ *Receptor active-inactive conformation*

Additional evidence of the two state receptor models (presented in section 2.2.6) comes from the β_2 adrenoceptor crystal structures. The β_2 adrenoceptor has been crystalized with both an agonist (BI-167107) and with an inverse agonist, (carazolol) (Rasmussen, et al. 2011b; Cherezov et al. 2007). Thus, it is possible to appreciate the difference between the "active" and an "inactive" conformations, respectivelly (Figure 8) (Rasmussen et al. 2011a).

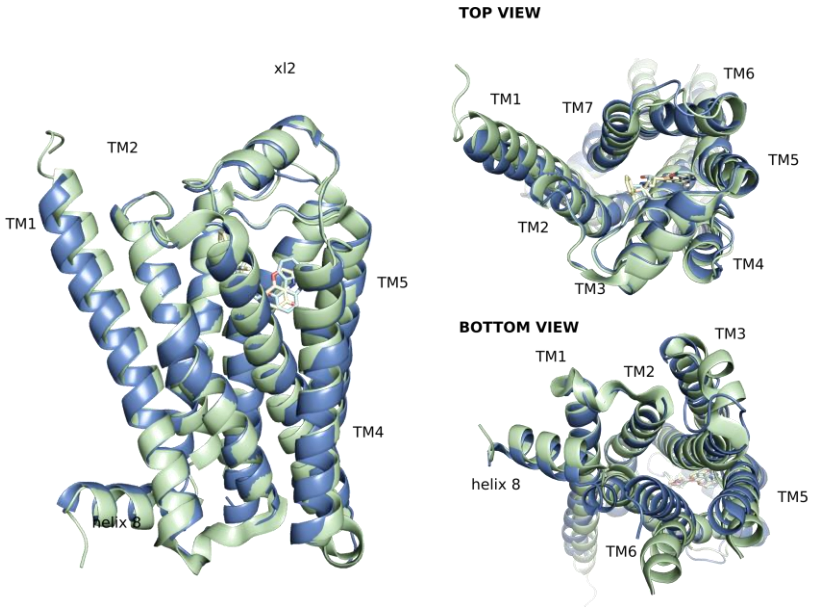


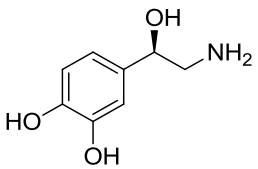
Figure 8 β_2-adrenoceptor in the active and inactive conformation. In blue β_2 adrenoceptor in the inactive conformation, with carazolol an inverse agonist pdb code 2HR1; in green β_2 adrenoceptor in the active conformation with BI-167107, pdb cose 3P0G. Adapted from Rasmussen et al. 2011a.

The overall root mean square deviation (rmsd) of the two receptor structure is less the 1Å (Rasmussen et al. 2011a). The largest differences are in the cytoplasmic site of the receptors, where TM6 and TM5 moves outward and away from the central core while TM7 and TM3 move inward. These movements break the conserved salt bridge (known to be important for activation) between D5.50 - R3.49, thus allowing a clockwise rotation TM6 of about 11Å. In contrast to the changes in the cytoplasmic domain, the changes in the binding pocket are minimal (Rasmussen et al. 2011b).

2.4.2. α_2-adrenoceptors

Adrenoceptors are GPCRs that bind endogenously adrenaline and noradrenaline (in U.S: epinephrine and norepinephrine) (Figure 9).

Noradrenaline



Adrenaline

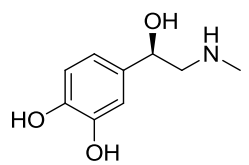


Figure 9 2D structure of noradrenaline and adrenaline.

Adrenoceptors are the main mediators of the sympathetic nervous system, regulating many physiological functions, such as the increase in heart rate, digestion functions, or altering the respiratory rate producing what is known as fight-or-flight responses. Adrenoceptors were divided into three main classes α_1-, α_2-, and β- adrenoceptors by Ahlquist in 1948 based on their affinity to different catecholamines and the different effect observed in the smooth muscles. A further classification divides the three classes in subtypes. Both α_1 receptors and β_2 receptors are expressed in the vascular system and bind to adrenaline and noradrenaline. Vasoconstriction occurs after stimulation of the α_1 receptor while the vasodilatation is caused by the binding of these catecholamines to β_2 receptor (Ahles & Engelhardt 2014).

In humans and mammals, there are three α_2-adrenoceptors subtypes: α_{2A}-adrenoceptor, α_{2B}-adrenoceptor and α_{2C}-adrenoceptor (Bylund et al. 1992). Based on classical physiological functions, α_2-adrenoceptors are considered presynaptic receptors that regulate, with negative feedback, the additional release or adrenaline or noradrenaline (Langer 1974). α_2-adrenoceptors mostly coupled with $G_{i\alpha}$ or $G_{o\alpha}$, inhibit the adenylate cyclase causing the reduction of cAMP in the cell, which then activates a receptor-operated K^+ channel and blocks the Ca^{2+} voltage channel (Limbird 1988). Thanks to molecular cloning, ligand binding, and anatomical and functional studies, it was possible to show that α_2-adrenoceptors are also expressed post-synaptically and extra-synaptically (Perälä et al. 1992; Scheinin et al. 1994).

The α_{2A}-adrenoceptor is composed of 450 amino acids, the α_{2B}-adrenoceptor of 447, and the α_{2C}-adrenoceptor of 462 (Xhaard et al. 2006). The major differences in the amino acids composition are present in the N-terminus (differing for the number of amino acids 28, 7, and 46 respectively A, B, and C), in the third intracellular loop and in the C-terminus (Xhaard et al. 2006). Additionally, the predicted glycosylation sites varies as the N-terminus of α_{2B}-adrenoceptor is much shorter than the other subtypes and cannot accommodate the two glycosylation sites present in the other receptors. The palmoytilations site is an important anchor for the protein to the membrane. This site is conserved in both α_{2A}-adrenoceptor and α_{2B}-adrenoceptor but not present in α_{2C}-adrenoceptor where the cysteine is replaced with a phenylalanine (Figure 10).

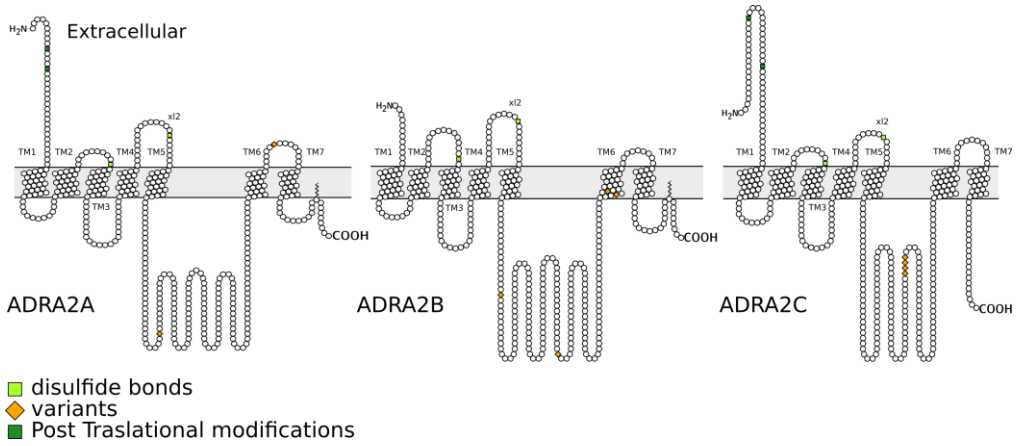


Figure 10 The α_2-adrenoceptors subtypes. The figure was generated using Protter (Omasits et al. 2014) and then modified.

The overall sequence identity between the three subtypes (Figure 11). It shows 170 conserved amino acids that represent the 55%, 59%, and 52% of the full sequence of α_{2A}-adrenoceptor, α_{2B}-adrenoceptor, and α_{2C}-adrenoceptor respectively. If considering only the amino acids surrounding the binding cavity the conservation is even higher with 25 conserved amino acids over the total of 30 (Table 2).

Table 2 Sequence variation of the amino acids predicted in the binding cavity for the three subtypes. In bold font the variable positions.

Amino acids location in the binding site

	2.53	2.57	3.25	3.28	3.32	3.33	3.36	3.37	4.52	4.56	4.60	5.38	**5.39**	5.42	**5.43**	5.46
α_{2A}	V	V	C	Y	D	V	C	T	I	I	P	Y	**I**	S	**C**	S
α_{2B}	V	V	C	Y	D	V	C	T	I	I	P	Y	**V**	S	**S**	S
α_{2C}	V	V	C	Y	D	V	C	T	I	I	P	Y	**I**	S	**C**	S

Amino acids location in the binding site

	5.47	6.44	6.48	6.51	6.52	6.55	7.39	7.42	7.43	7.45	**xl2.49**	xl2.50	**xl2.51**	**xl2.52**
α_{2A}	F	F	W	F	F	Y	F	G	Y	N	**R**	C	**E**	**I**
α_{2B}	F	F	W	F	F	Y	F	G	Y	N	**Q**	C	**G**	**L**
α_{2C}	F	F	W	F	F	Y	F	G	Y	N	**Q**	C	**Q**	**L**

α_2-adrenoceptors are localized in both the central nervous system, peripheral nervous system, and peripheral tissues (like smooth muscles) (Saunders & Limbird 1999). A precise pharmacological function of each subtype is difficult to define, largely due to the lack of subtype-selective probes. Anatomical data on expression have been used to link specific physiological function of the subtypes. α_{2A}-adrenoceptors and α_{2C}-adrenoceptors are predominantly expressed in the central nervous system while the α_{2B}-adrenoceptor is expressed predominantly in the vasculature. In addition to the negative regulation of noradrenaline and adrenaline neurotransmitter feedback, in some

cases the α_{2A}-adrenoceptor and α_{2C}-adrenoceptor act as heteroreceptors inhibiting the release of other neurotransmitters like dopamine or serotonin (Bücheler et al. 2002; Scheibner et al. 2001). They regulate the pain perception (antinociception), sedation and hypnosis, behavioural functions (inhibiting the processing of sensory information, thus can be used in schizophrenia, post-traumatic stress, attention deific disorder and drug withdrawal), and cardiovascular (with a hypotensive and bradicardiac function) (Hein 2006).

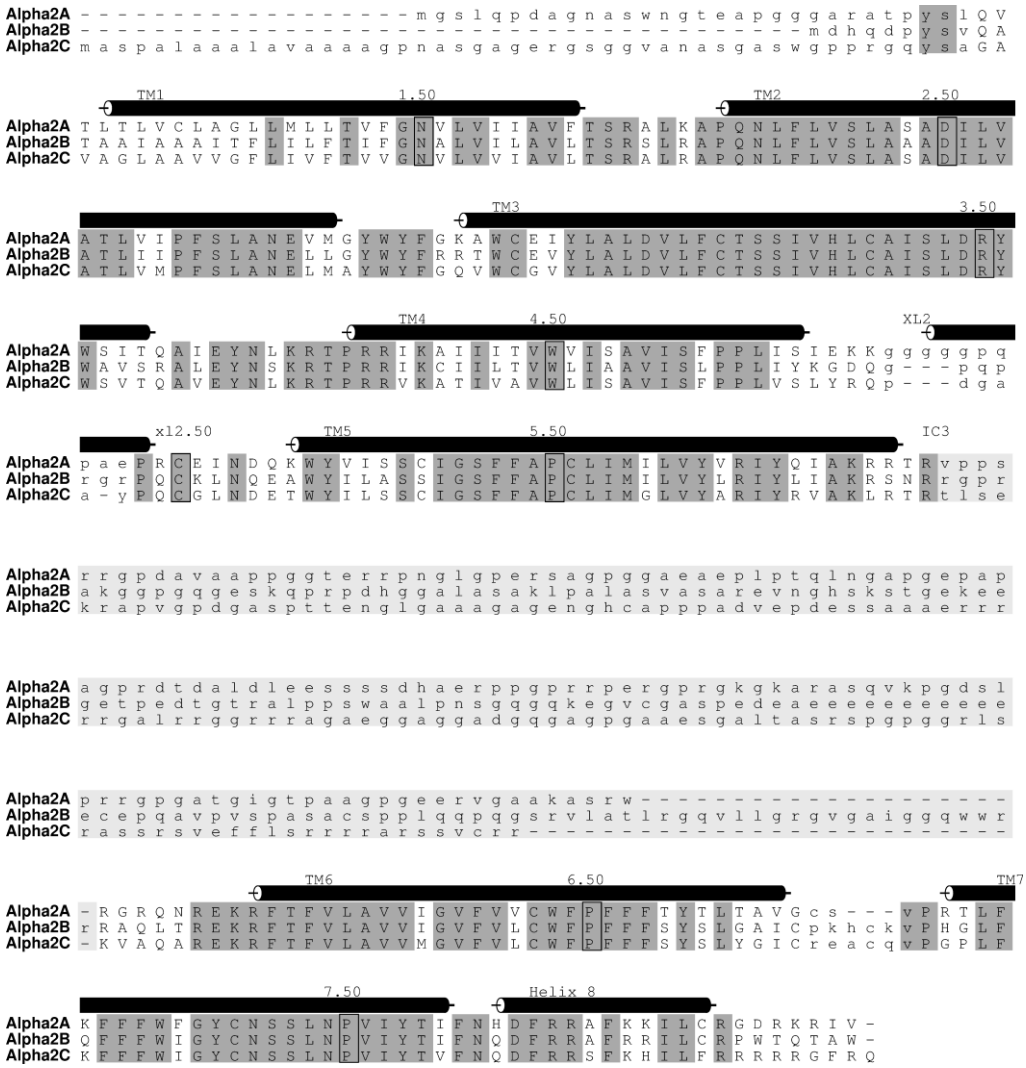


Figure 11 Alignment of the α_2-adrenoceptors subtypes.

2.4.3. Deciphering α_2-adrenoceptors molecular features

The pharmacological differences of α_2-adrenoceptors subtypes have been difficult to elucidate, in fact the majority of the drugs used are not subtype selective and can cause important side effect (Gyires et al. 2009).

In Finland, Juvantia Pharma Ltd discovered several compounds that showed selectivity of 1000-fold to the α_{2C}-adrenoceptor and some to the α_{2B}-adrenoceptor (Höglund et al. 2006). An example is the ligand presented in patent number US6521632B2 an α_{2B}-adrenoceptor selective, and fipamezole an α_2-adrenoceptor selective antagonist (Michel et al. 1990). Fipamezole is currently licenced to Santhera pharmaceuticals and succeeded as a drug candidate for the treatment of levodopa-induced dyskinesia in Parkinson's disease (Savola et al. 2003).

Currently dexmedetomidine and guanfacine are new α_2-adrenoceptor selective agonist but have shown to have also affinity towards other GPCRs (Lee et al. 2013; Lowry & Brown 2014; Savola et al. 2003). From the industrial point of view, small molecules like medetomidine, an α_2-adrenoceptor agonist, has been developed as antifouling and used now for its sedative properties as anaesthetic for small animals (Lind et al. 2010).

In the last 20 years in Finland, much as been done to understand the important residues in ligand binding to the α_2-adrenoceptors subtypes (Salminen et al. 1999; Nyrönen et al. 2001; Peltonen et al. 2003; Xhaard et al. 2005; Laurila et al. 2007). Many different techniques, ranging from biophysical/biochemical methods to molecular modelling in combination with molecular pharmacology, have been used to map the amino acids exposed in the binding pocket of the active (agonist-bound) and inactive (antagonist-bound) conformations. In particular, covalent binders such as phenoxybenzamine, chloroethylclonidine (CEC), and 2-aminoethyl methanethiosulfonate (MTSEA) were used in conjunction with receptor constructs bearing engineered cysteines (Frang et al. 2001; Salminen et al. 1999; Marjamäki et al. 1999).

As a result, molecular interaction of the catechol ring were identified to be quite similar to the ones found in the β_2-adrenoceptors bound to isoprenaline; identifying hydrogen bonds between the hydroxyl groups of the catechol and with S5.42 and S5.46 (with meta-OH and para-OH respectively (Peltonen et al. 2003; Xhaard et al. 2006). Additionally, it was suggested the β-hydroxyl of the aspartate D3.32 is responsible for an additional hydrogen bond with the R-enantiomers (Nyrönen et al. 2001). With the use of docking simulation seven additional interactions were predicted to be important

for agonist binding to the α_2-adrenoceptors: V3.33, T3.37, C5.43, F5.47, Y6.55, F7.38, and F7.39 (Nyrönen et al. 2001; Peltonen et al. 2003).

Additional studies have been conducted to better define the key residues involved in antagonist binding. It has been suggested, with the use of docking techniques, that the binding site of an antagonist is larger than the one for an agonist, as typically antagonists are larger the agonists (Xhaard et al. 2005). Antagonist binding showed important hydrophobic interactions with TM6 and TM7 (F6.44, W6.48, F6.51, F6.52, and F6.53) (Gentili et al. 2004). Additionally, xl2.49 and xl2.51 (as well as 5.43) were found to be important in yohimbine binding (Laurila et al. 2007).

3. Aims of the Study

The main goal of this study is to identify novel modulators acting on ABCC2 transporters and α_2-adrenoceptors subtypes and to understand their mode of action. With this purpose screening approaches and chemoinformatic analysis were used to identify novel modulators. In-depth experimental studies were performed to understand the interactions between ligands and proteins and their mode of action.

- **Find novel compounds for ABCC2 and the α2-adrenoceptors subtypes using screening approaches**

  - Acquire insights into the binding profile, "instant SARs" and identify new modulators of ABCC2. *Publication II and Unpublished results*
  - Identify α_2-and α_{2B}-adrenoceptors selective compounds. *Publication IV*

- **Perform chemoinformatics analyses of screening data on ABCC2 and the α_2-adrenoceptors subtypes**

  - Analyze, map the biological activities and represent the compound property space (similarity clustering). *Publication II and Publication IV*
  - Extract molecular descriptors important for discriminating ABCC2 inhibitors from inactive molecules. *Publication II*

- **Conduct an in-depth study of the mode of action of the compounds interacting with ABCC2 and the α_2-adrenoceptors subtypes**

  - Identify the specific effects of compounds on different ABCC2-mediated probe transport. *Publication I and Publication II*
  - Explain the 10- to 100- fold differences in binding differences among the α_{2A}, α_{2B}- and α_{2C}- adrenoceptor subtypes. *Publication III*

- **Understand the relationships between compound structures and their biological activity using homology modelling**

  - Identify the molecular interactions between α_2-adrenoceptors and a set of antagonists. *Publication III*
  - Glean further insights into the interaction between ABCC2 and inhibitors. *Unpublished results*

4. Materials and methods

In this section, a short summary of the materials and methods used is presented. The specific and detailed descriptions of the methods are found in the original Publications I-IV.

4.1. Materials

❖ *Chemical compounds*

A small set of commercially available compounds were tested in Publication I and Publication III. In Publication I, eight compounds were selected from literature as known ABCC2 modulators, and used for the probe comparison in the vesicular transport assay (described below). In Publication III, nine known antagonists, selected as known α_2-adrenoceptors antagonist, have been evaluated for α_2-adrenoceptors chimera subtype binding affinity.

In Publication II and Publication IV, a screening-type approach was used to identify active molecules. In Publication II, 432 compounds were tested with a vesicular transport assay. These compounds were of built using combinatorial and heterocyclic chemistry obtained through the collaboration of Professor Peter Wipf from the University of Pittsburgh, Center for Chemical Methodologies and Library Development.

In Publication IV, 17,798 compounds were screened, 2112 of these molecules could be classified as FDA approved drugs, and the rest were defined as general compounds derived from the ChemDiv (San Diego, CA, USA), ChemBridge (San Diego, CA, USA), Tripos Discovery Research (now Exelgen Dis- covery, Bude, UK) and the MicroSource Spectrum collection (MicroSource Discovery Systems, Inc., Gaylordsville, CT, USA).

4.2.Methods

4.2.1. Experimental methods

❖ *Protein expression and membrane preparation*

ABCC2 was expressed in a suspension of *Spodoptera frugiperda* insect ovarian cells (Sf9), while the α_2-adrenoceptors subtypes were expressed in adherent Chinese hamster ovary cells (CHO) by the collaborators. Membrane preparation was quite similar for both proteins. The collected cell pellet was suspended in a hypotonic lysing buffer and homogenized, and subsequently centrifuged to remove nuclei and aggregates. Additional steps of centrifugation and re-suspension were needed to isolate the membrane fraction of the insect cells. Vesicles used in Publication I and Publication II were then prepared from a membrane suspension by passing through a 27-gauge needle.

❖ *Vesicular transport assay*

Vesicular transport assay (VT-assay) is the simplest method that can be used to predict interactions of compounds with transporters, as ABCC2, and thus to assess the liability of compounds for potential transporter-mediated DDIs. When combined with a detection technique, such as mass spectroscopy or when using labelled substrates, the VT-assay can also be used to give further insights into the actual transport. Inverted vesicles create a direct access point for the substrate to the transporter binding site (Brouwer et al. 2013)

The VT-assay functions by measuring the ATP-dependent uptake of substrates into the vesicles. Uptake is detected by fluorometry, radioactivity, or mass spectroscopy (Figure 12, left side). The assay usually is used to indirectly measure the modulatory effect of the tested compounds on the transport of the detectable substrate probe (Figure 12, right side).

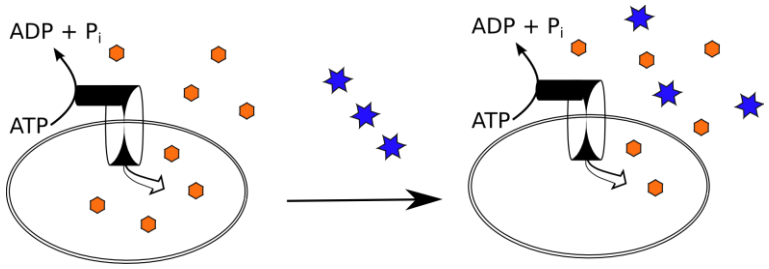


Figure 12 Vesicular transport assay. The ATP dependent uptake of the probe (orange) in the vesicle is measured. A modulator (blue) alters is added and the ATP depended transport of the probe(right side).

After the test compound is added, the change of active transport of the probe is measured. Inhibitors decrease the transport of the probe, while stimulators will increase it. Compounds inhibiting/stimulating the ABCC2 transport may or may not be substrates, as they are not necessarily transported by the protein. It has been proposed that ABCC2 can co-transport compounds with glutathione; therefore, it was added to the assay (Rappa et al. 1997).

Several commercial assays, optimized for vesicular transport with different ABC transporters are available. For the ABCC2 VT-assay, three substrate probes have optimized these are : leukotriene C4 (LTC4), β-estradiol 17-(β-D-glucuronide (EG), and 5 (and 6)-carboxy-2',7'-dichlorofluorescein (CDCF) (Figure 13) (Chen et al. 1999; Bodó et al. 2003; Heredi-Szabo et al. 2008). In Publication I, VT-assays with all three probes were used; while only EG and CDCF assays were used in Publication II.

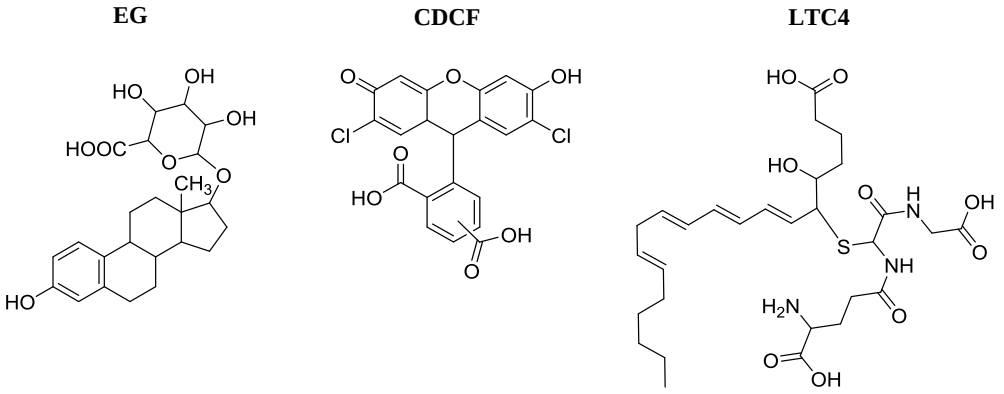


Figure 13 ABCC2 VT-assay probes. EG, β-estradiol 17-(β-D-glucuronide; CDCF 5 (and 6)-carboxy-2',7'-dichlorofluorescein; LTC4, leukotriene C4.

❖ *Competition binding assay*

The competition binding assay was performed on α_2-adrenoceptors subtypes by the collaborators using two antagonist radioligands, RX821002 (in Publication III) and RS79948-197 (in Publication IV). Briefly, before performing the assay, the equilibrium dissociation constant (K_d) and receptor binding site density (B_{max}) were calculated to define the affinity of the radioligand and specific binding. In Publication III, the affinity of the radioligand was first determined for each of the mutated receptor with a saturation assay.

In a competition binding assay, the displacement of the labelled ligand from the orthosteric site is detected. Ligands that bind to the orthosteric site will compete with the radioligand for the site. With this experiment, it is not possible to define the functional effect (agonist or antagonist) of the competing ligand. If the function effect is needed a functional assay has to be performed. In Publication IV, in addition to the competition binding assay, a functional [35S] GTPγS assay was performed. Such assay identifies the activation of the G-protein signalling pathway, specifically, it detects the guanidine nucleotide exchange (GDP > GTP) in the G-protein in the agonist activation (Peltonen et al. 1998).

❖ *Screening*

Screening, of a small/medium sized library was performed with either the VT-assay or with a competition binding assay. Generally, screening is often an automated biochemical assay set up that is used to assess the activity of a set of compounds. In a screening approach, a large set of compounds are tested initially, but usually only a set of active compounds called "hits" are selected for further analysis. With this approach, it is possible to maximise the number of tested compounds and minimize the costs. Screening performance can be evaluated statistically. The most common parameters that are calculated include the Z', which is a parameter that defines if the assay response is large and clear enough to be seen in the assay setup; signal to background (S/B ratio), and signal to noise (S/N ratio) (Eq. 12, Eq. 13, Eq. 14) (Zhang et al. 1999). The parameters are calculated as following, considering X as the mean of the calculated values, SD the standard deviation, B for minimum, and S for maximum accordingly.

Z'	$Z' = 1\frac{3SD_S+3SD_B}{\lvert X_S-X_B\rvert}$	Eq. 12
Signal to background ratio	$S/B = \frac{X_S}{X_B}$	Eq. 13
Signal to noise ratio	$S/N = \frac{X_S-X_B}{\sqrt{SD_S^2-SD_B^2}}$	Eq. 14

Screening approaches have been used with the aim of characterising ABCC2 modulators in Publication II and identifying selective α_2-adrenoceptors ligands in Publication IV.

Additionally, in Publication IV, a miniaturization binding assay was used. Miniaturization reduces the amount of biological and chemical reagents used per assay, thus reducing costs. However, it may result in the loss of hit compounds due to low signal-to-noise ratios.

❖ *IC_{50} calculation and curve fitting*

IC_{50} is the molar concentration of an unlabeled agonist or antagonist that inhibits the binding of the radioligand by 50%; or in the case of transporters: the molar concentration that inhibits the probe transport by 50%. It is normally calculated from concentration-response curves (Figure 15) of inhibitors (Eq. 15).

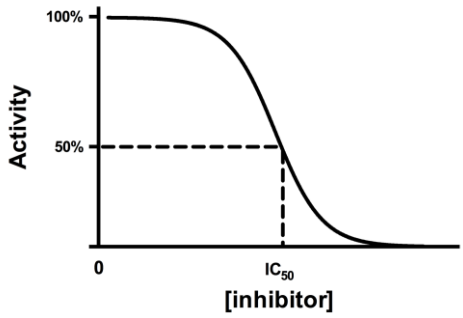


Figure 14 IC_{50} concentration-response curve

$$Activity = min + \frac{max-min}{1+\left[\left(\frac{[inhibitor]}{IC_{50}}\right)\right]^{-\beta}} \quad \text{Eq. 15}$$

The *Hill coefficient* or *β factor* can be used to "better fit" the data to the Michaelis-Menten equation where V_0 is the transport rate at a specific moment; V_{max} is the maximum transport rate; S is the substrate concentration; K_m is the substrate concentration at half of V_{max}. as following (Eq. 16)

$$V_0 = \frac{V_{max} * S^{\beta}}{{K_m}^{\beta} + S^{\beta}} \qquad \text{Eq 16}$$

In enzyme kinetics the *Hill coefficient* is correlated with cooperativity in binding that can be positive (>1) or negative (<1). This coefficient can define the number of cooperative binding sites but cannot indicate the number of functionally important binding sites. For competitive inhibitors the *Hill coefficient* should be 1 (Weiss 1997). In the case of transporters it has been suggested that these values are useful for fitting the experimental data but do not represent physical parameters (Bentz et al. 2005).

For competitive inhibitors, additionally, it is possible to convert the IC_{50} to K_i utilizing the Cheng-Prusoff equation (Eq. 17). The equation can be used only in the case of competitive inhibitors and when the concentration of substrate is much higher than the IC_{50}.

$$K_i = \frac{IC_{50}}{1 + \frac{S}{K_m}} \qquad \text{Eq. 17}$$

4.2.2. Computational methods

❖ *Sequence alignment and homology modelling*

Computational methods have been developed to estimate the 3D structure of proteins when no crystal structure is available. These methods are divided in two major classes: template based (homology) modelling and *de novo* modelling. Of these methods homology modelling (or comparative modelling) is considered to be the more accurate and more commonly method used in drug discovery. The basic hypothesis that drives this method is the observation that proteins sharing a common ancestor (i.e. homologous proteins) exhibit the similar structure or fold (Chothial & Lesk 1986).

This method depends on the use of the 3D coordinates of a homologous protein template structure and the sequence of the target protein. Different methods have been developed to build homology models and all share similar steps: 1. Identify known structures and align them to the target protein; 2. Define suitable template structure based on the alignment and the quality of the structure; 3. Build a model by replacing the atomic positions of the template with the amino acids present in the alignment; and 4. Refine the model (Schmidt et al. 2014).

There are several approaches used to align the target sequence to the template structure. Sequences can be aligned either pairwise, when the alignment is done with only the target and the protein, or with multiple alignments, where many homologous protein sequences are used. A structural alignment can be done for two or more 3D structures of proteins with a similar fold to identify stretches of amino acids that correspond to the structurally conserved parts of the protein. Generally, structural alignments are preferred as the structures reveal the real correspondence for the amino acids in the compared structures.

In Publication III and Publication IV, the homology models for the α_2-adrenoceptor subtype were built based on β_2-adrenoceptor template structures. The target sequence was aligned to the structural alignment of more crystallized GPCRs.

The homology model of human ABCC2 was built using mouse mdr1 as the template presented in the Unpublished results. The alignment was derived from a multiple alignment of the NBDs and TMDs of all 48 human ABC proteins (sequences retrieved from the Uniprot KB data base, The UniProt Consortium)

❖ *Molecular docking*

Molecular recognition is a key feature in many biological processes and molecular docking aims to predict these interactions. Molecular docking is normally composed of two parts, the estimations of favourable low-energy binding poses and the ranking/scoring of these poses. Generally, many binding poses are obtained for one ligand, and thus, the poses have to be evaluated. The scoring of binding poses involve four general categories (Liu & Wang 2015): physics-based methods, empirical scoring functions, knowledge-based potentials, and descriptor-based scoring functions. Many scoring functions do not optimally define structural water molecules and the enthalpy contributions of the interaction are overlooked (Sousa et al. 2013).

Docking programs are considered to perform relatively well in predicting ligand poses in comparison to their scoring and ranking function. Docking with GOLD™ (Jones et al. 1997) was used to dock antagonists in Publication III, and 2,2,-bisepigallocatechin digallete, in Publication IV. In both docking simulations, the aspartic acid D3.32 was used to define the central point of the binding cavity. At the time of the work, flexible protein docking was not available. To overcome the lack of this technology, many different homology models of the protein were built mimicking the flexibility of the side chains.

ShaEP has been used for the ranking and selecting of the best docking pose of α_2-adrenoceptor antagonist in Publication III. ShaEP is a software that overlays molecules based on their shape and electrostatic potential (Vainio et al. 2009). All docked poses presented in Publication III, were ranked based on the comparison of crystalized ligand, carazolol, bound to β_2-adrenoceptor.

❖ *Chemical scaffolds*

A chemical scaffold is the common core structure, or substructure, which characterizes a group of molecules (Schuffenhauer et al. 2007). Traditionally, molecules sharing the same scaffold are presumed to have the same synthetic pathway, e.g. combinatorial chemistry libraries. Scaffolds are generally a defined ring-system that keeps the substituents in their position (Ertl et al. 2006).

The Markush structures were first used in a patent clam in 1924 and are now the most common way to represent molecules that share the same scaffold. In such representation the common core of the molecules is defined and the substitute

variations, the individual functional groups of each molecule, are given separately as R-groups (Figure 15).

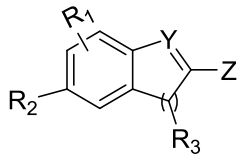


Figure 15 Markush structure. The first Markush structure published in the patent clam in 1924.

A variant of the Schuffenhauer rules was used to divide the combinatorial library used in Publication II. Additionally, all the molecules have been described as Markush structures. Hierarchical clustering using maximal common substructure was used to classify hit compounds selected in Publication IV.

❖ *Structure- activity relationships*

The analysis of structure-activity relationships (SAR) is the central core of medicinal chemistry. The association between the biological activity of molecules and the chemical properties is fundamental to identify, optimize, and evaluate new potential drug targets. The assumption of any SAR method is that similar molecules have similar activity, and that it is possible to identify the crucial determinants correlating with the biological activity. Many methods have been developed, focusing on different chemical descriptors of the molecule or on different algorithms to correlate the descriptors to the activity.

Supported vector machine (SVM) (Cortes & Vapnik 1995) and XY-fused Kohonen Networks neuronal network (Kohonen 1982) model were built to classify molecules into inhibitors and inactive molecules in Publication II. Both machine learning methods use an algorithm that recursively learns from the data identifying patterns for the classification. The SVM classification separates the data in two or more classes while the XY-fused Kohonen Networks defines the two classes based on self-organizing maps.

Binary classification test was performed to validate the models. Initially, the number of true positives (TP), true negatives (TN), false positives (FP), and false negatives (FN) are defined; consequently the accuracy, sensitivity, and specificity are calculated (Eq.

18, Eq. 19, Eq. 20). In addition to these parameters the Matthews correlation coefficient (MCC) was calculated (Eq. 21).

Accuracy (%) $$ACC\ (\%) = \frac{TP+TN}{TP+FP+TN+FN}$$ Eq. 18

Sensitivity (%) $$SE\ (\%) = \frac{TP}{TP+FN}$$ Eq. 19

Specificity (%) $$SP = \frac{TN}{TN+FP}$$ Eq. 20

$$MCC = \frac{TP \times TN - FP \times FN}{\sqrt{((TP+FP) \times (TP+FN)(TN+FP) \times (TN+FN))}}$$ Eq. 21

❖ *Pharmacophore*

The term pharmacophore was first defined by Ehrlich in 1909 as the molecular fragment that carries the essential features for a drug's biological activity. A pharmacophore can be created in a ligand-based manner, where a set of active molecules are superimposed and the common chemical features for activity are extracted, or in a structure-based manner by proposing possible interaction points between ligand and target (Yang 2010). Generation of ligand-based pharmacophores starts with the identification of all possible conformations of the active molecules, which are then aligned. The alignment can either:

1. be point-based using least-square fitting of atoms, fragments, or chemical features
2. properties-based, using molecular field descriptors and aligning them using Gaussian functions.

Pharmacophores of ABCC2 inhibitors were built in a ligand-based manner using Discovery Studio (Accelrys Software Inc., Discovery Studio Modeling Environment, Release 4.0, San Diego: Accelrys Software Inc., 2013.). The automatically generated conformations of the ligands were aligned using a scaffold superimposition.

5. Summary of main results

Results are presented according to the aims as in Section 3; unpublished results will be presented in the following Section (6).

- **Find novel compounds interacting with ABCC2 and the α_2-adrenoceptors subtypes using screening approaches**

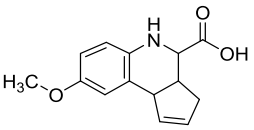
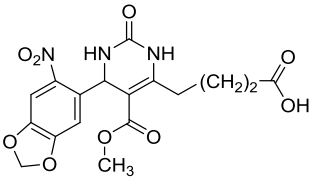
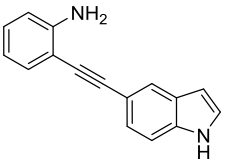
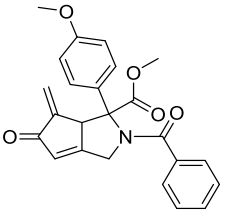
In Publication II, a small-size library of 432 compounds was used to acquire insights into the activity profiles of ABCC2 modulators. The library was initially tested at one data point at 80µM with two probes CDCF and EG (primary screening). From the primary data, we analysed the modulatory effect calculated as % inhibition of probe transport (%I) and grouped the results into five different classes (inhibitors, borderline inhibitors, inactive, borderline stimulators, and stimulators). Considering only inhibitors (I% < 50) and stimulators (%I > 150), 22% of the 432 tested compounds showed a modulatory effect.

After the initial screening 86 compounds were selected for further analysis; initially three concentration points were calculated and from these 50 were selected for IC_{50} calculations. In total, 25 EG and 38 CDCF inhibitors were identified, of these 23 inhibited both probes. Additionally, of these 23 shared inhibitors, 16 had an IC_{50} lower than 30µM for at least one probe, four molecules are presented in Table 3.

Only few probe selective modulators have been identified. One clear probe selective inhibitor **3j** (50-fold difference between CDCF and EG inhibition) and another four (**4d**, **4f**, **17** and **21**) with potentially different type of modulation (with some uncertainties due to the only partial concentration-response curve) are presented in Table 4.

Table 3 A selections of four ABCC2 inhibitors. 1l, 2u, 6 and **29** with their relative calculated IC_{50}, numbered as in Publication II.

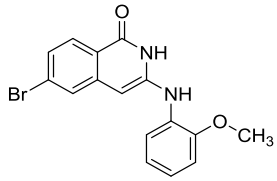
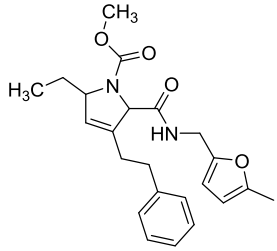
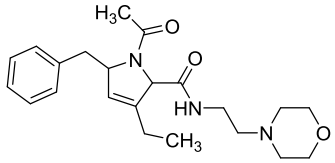
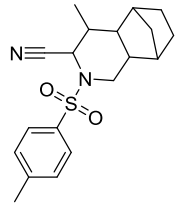
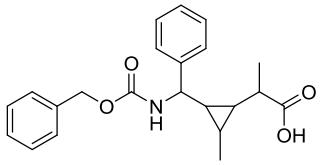
ABCC2 inhibitors

		CDCF		EG	
#	**Structure**	**IC_{50}, µM * (95% CI)**	**Hill slope (95% CI)**	**IC_{50}, µM * (95% CI)**	**Hill slope (95% CI)**
1l		12 (10 - 15)	-1.4 (-1.8, -1.0)	9 (4 - 22)	-0.9 (-1.5, -0.3)
2u		57 (37 - 87)	-1.4 (-2.2, -0.7)	15 (4 - 57)	-1.3 (-3.3, -0.7)
6		23 (14 - 38)	-1.5 (-2.4, -0.5)	weak inhibitor [a]	
29		7 (5 - 11)	-1.1 (-1.4, -0.7)	inhibitor [b]	

[a]modulatory effect estimated from three concentrations only (400µM,80µM,16µM); [b]4-parameter logistic model curve could not be fitted. * calculated at 400µM, 80µM,16µM,3.2µM, 0.64µM and 0.128µM.

Table 4 The five probe selective molecules. Molecules are numbered as in Publication II.

Probe selective molecules

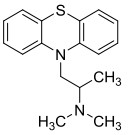
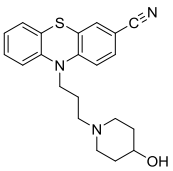
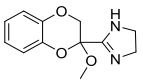
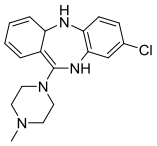
#	Structure	CDCF		EG	
		IC_{50}*, µM (95% CI)	**Hill slope (95% CI)**	**IC_{50}*, µM (95% CI)**	**Hill slope (95% CI)**
3j		6 (2 - 23)	-0.9 (-1.5, -0.2)	305 (220 - 423)	-1.1 (-1.7, -0.4)
4d		37 (14- 97)	-0.6 (-0.9, -0.3)	weak inhibitor [a]	
4f		inactive [a]		70 (18 - 283)	-1.0 (-2.1, 0.1)
17		stimulator		inactive [a]	
21		220 (173 - 279)	-1.6 (-2.1, -1.1)	stimulator [a]	

[a]modulatory effect estimated from three concentrations only (400µM,80µM,16µM); [b]4-parameter logistic model curve could not be fitted. * calculated at 400µM, 80µM,16µM,3.2µM, 0.64µM and 0.128µM.

In Publication IV, a mid-sized library (of 17952 molecules) was screened with a miniaturized competition binding assay to identify α_{2B}– adrenoceptors selective compounds. The library could be divided in two subset, approved drugs, and general compounds (defined as in section 4.1). From the initial screen, tested with a single concentration of 10µM concentration against the human α_{2B}-adrenoceptor, 176 preliminary hits were selected. These preliminary hits were then retested at 1µM concentration so only high affinity molecules could be identified. After this second selection, 93 molecules were selected to be further characterized. The 93 molecules showed to have an affinity in the low µM- nM range towards the α_{2B}-adrenoceptor (defined as K_i values). Of these molecules, twelve molecules were approved drugs one of the three were of the general subset. Of the twelve molecules selected from the approved drugs subset four were α_{2B}-adrenoceptor selective, six favoured α_{2A}-adrenoceptor, while two were selective for α_{2C}-adrenoceptor (Table 5). Interestingly, promethazine was not known previously to be active towards α_2-adrenoceptors and showed to be α_{2B}-adrenoceptor selective (Table 5). In addition to the selective approved drug molecules two new α_2-adrenoceptors selective compounds were identified from the general subset (Table 5).

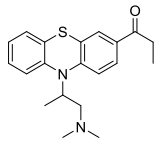
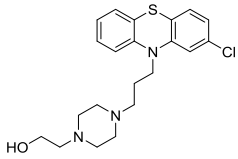
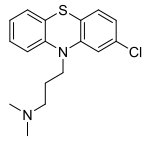
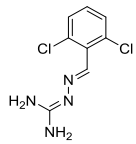
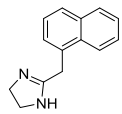
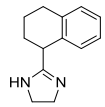
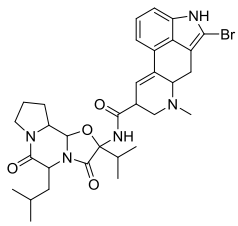
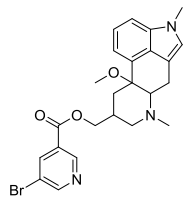
Table 5 Subtype specific molecules (continues on the following pages 49-50).

Subtype selective molecules

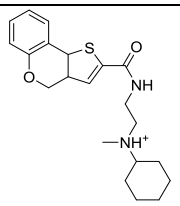
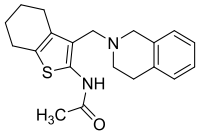
		α_{2B}	**α_{2A}**	**α_{2C}**
Name	**Structure**	**K_i, nM (95% CI)**	**K_i, nM (95% CI)**	**K_i, nM (95% CI)**
Promethazine		160 (87-286)	n.d[#]	n.d[#]
Periciazine		5.2 (1.5-23)	307 (111-715)	121 (49-287)
2-Methoxyidazoxan		16 (6-35)	0.67 (0.28-1.4)	5.1 (2.6-8.5)
Clozapine		31 (13-68)	132 (40-308)	20 (4-26)

[#]IC_{50} was not calculated as the percentage inhibition at 1µM higher than 50%.

Continuation of Table 5.

		α_{2B}	α_{2A}	α_{2C}
Name	**Structure**	**K_i, nM (95% CI)**	**K_i, nM (95% CI)**	**K_i, nM (95% CI)**
Propiomazine		74 (42-133)	747 (423-1318)	813 (89-633)
Perphenazine		95 (23-252)	917 (294-3468)	285 (89-633)
Chlorpromazine		151 (82-307)	1762 (953-3259)	442 (248-819)
Guanabenz		349 (94-1013)	21 (11-36)	236 (123-688)
Naphazoline		476 (109-1613)	10 (4-25)	410 (237-708)
Tetrahydrozoline		1034 (584-1830)	44 (19-86)	n.d
Bromocriptine		1260 (321-6720)	98 (35-227)	1770 (388-5866)
Nicergoline		2699 (1425-5608)	185 (49-424)	362 (185-722)

Continuation of Table 5.

		α_{2B}	α_{2A}	α_{2C}
Name	**Structure**	**K_i, nM (95% CI)**	**K_i, nM (95% CI)**	**K_i, nM (95% CI)**
ChemDiv C712-1339		845 (367-2027)	75 (22-220)	124 (44-287)
ChemDiv 1724-0247		923 (458-2081)	34 (18-62)	142 (86-250)

- **Perform chemoinformatics analyses of collected screening data on ABCC2 and the α_2-adrenoceptors subtypes**

In Publication II, SOM maps are used to map the activities of the modulators on the chemical space. Such maps provide a simple way to visualise the activity data. From the maps it was observed that modulators and inactive molecules cluster in different chemical spaces. Additionally, the data obtained from the primary screen was used to build a supported vector machine (SVM) predictive model. The tested SVM model was able to discriminate between inactive compounds and inhibitors and predict the correct class with an accuracy of 66% (calculated with an external dataset). As a result important discriminative features between inhibitors and inactive molecules have been identified, being the number of rings, the solubility, and the hydrophobicity.

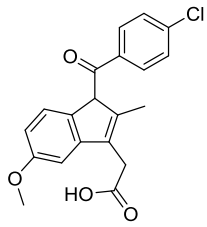
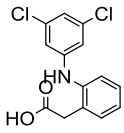
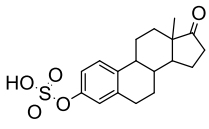
In Publication IV, a hierarchical classification tree of active compounds was constructed identifying 28 distinct scaffold/groups; of these ten were new scaffolds active on α_2-adrenoceptors. Of these ten scaffolds, two were identified as interesting scaffolds to further develop for subtype selective molecules, thienochromenes (as in ChemDiv C712-1339) and pyridopymidines (as in ChemDiv 1724-0247) presented in Table 5. This scaffold tree was provided as a tool to facilitate the visualisation and the analysis of the screening results.

- **Conduct an in-depth study of the specific mode of action of the compounds interacting with ABCC2 and the α_2-adrenoceptors subtypes**

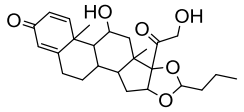
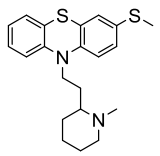
In Publication I, the VT assay (using either CDCF, LTC4, or EG as substrate probe) was utilised to decipher and characterise the specific effects of compounds on the ABCC2-mediated probe transport. Eight reference compounds selected as known ABCC2 modulators from literature were tested in the assay with the different probes. Three compounds had probe-independent effect; quercetin inhibited the transport of all three probes, while disopyramide and paracetamol did not effect at all the probe transport. Of the probe dependent modulators indometacin, diclofenac, and estrone-3-sulphate showed inhibition of CDCF and LTC4, while they stimulated EG transport. Budesonide and thioridazine inhibited EG transport, while they stimulated CDCF transport and did not affect LTC4 transport. In addition, the IC_{50} for inhibitors were calculated showing additional probe selectivity based on a different degree of inhibition of the probes (for example indomethacine inhibits both LTC4 and CDCF with IC_{50} of 246 and 75 respectively). The probe selective molecules are presented in Table 6.

Table 6 Probe selective compounds selected from Publication I. (continues on the following page).

Probe selective molecules

		LTC_4	**CDCF**	**EG**
Name	**Structure**	**IC_{50}, µM**	**IC_{50}, µM**	**IC_{50}, µM**
Indomethacin		246.6 ± 23.8	75.7 ± 12.9	Stimulates
Diclofenac		233.6 ± 47.5	177.3 ± 15.0	Stimulates
Estrone-3-sulfate		97.9 ± 19.4	122.2 ± 1.4	Stimulates

Continuation of Table 6.

		LTC_4	CDCF	EG
Name	**Structure**	**IC_{50}, µM**	**IC_{50}, µM**	**IC_{50}, µM**
Budesonide		Indifferent	Stimulates	60.3 ± 6.1
Thioridazine		Indifferent	Stimulates	40.9 ± 7.2

In Publication II, five possible probe selective molecules are presented in Table 4. Additionally, to further analyse the probe effect, primary screening results were compared between CDCF and EG modulation. The modulatory effect of the two assays showed an agreement of 91%, when considering the classification as inhibitors, inactive compounds, and stimulators. When the correlation was calculated between the percent inhibition of CDCF and EG, the correlation decreased to a 0.36. The probe effect here identified is not as common as it might be inferred from Publication I.

In addition, the robustness presented in supplementary material in Publication II shows that the standard deviation of the individual points calculated at the different concentrations was lower for the CDCF assay than for the EG assay. The average of the standard deviation ranged between 5-11% for the CDCF assay and 10-20% for the EG assay.

In Publication III, eight chimeric α_2-adrenoceptors were constructed with either N-terminus-TM1 swapped between the three subtypes or only the TM1 (maintaining the wild type N-terminus) with the aim of identifying subtype selective features. Nine known α_2-aderenoceptors antagonists have been used, and the affinities of these molecules to wild type receptors and chimeras have been defined with a competition binding assay. For three bulky antagonists (spiperone, spiroxatrine, and clorpormazine) the competition binding assay found a lower affinity, for α_{2A}-adrenoceptor when compared to the binding affinities to α_{2B}- and α_{2C}-adrenoceptor wild type. Chimeras with N-terminus-TM1 swapped between α_{2A}- and α_{2B}-adrenoceptors showed a significant increase of affinity for spiroxatrine and clorpormazine. Similarly, chimeras with N-terminus-TM1 swapped between α_{2A}- and α_{2C}-adrenoceptors showed the increase of affinity for all of the three antagonists. Additional experiments showed the involvement of the only TM1 in the ligand recognition, and not the N-terminus. As a

consequence of these experimental results it was shown the involvement of TM1 in bulky antagonist binding. These experimental results combined with the molecular modelling results suggested that TM1 does not directly affect the ligand bind, nevertheless influencing the binding indirectly.

- **Understand the relationships between compound structures and their biological activity using homology modelling**

In Publication III, homology modelling and docking simulation have been used to define the involvement of TM1 in the binding of three bulky antagonists. Homology models of the three subtypes were built using β_2-adrenoceptor as the template. A comparison of these new β_2-adrenoceptor based models were a compared with the rhodopsin based model previously published. The β_2-adrenoceptor based models showed a: 1. Shorter and wider binding cavity; 2. Longer TM1-TM5 distance (by 3 Å); 3. Presence of a α-helix extracellular loop position closer and deeper in the binding cavity; 4. The xl2.49 residue less exposed to the binding cavity; 5. The xl2.54 residue more exposed to the binding cavity. To better assess the interaction in the orthosteric site of the antagonist docking, simulations have been performed. After pose selection, using ShaEP, the docking poses showed interaction with both D3.32 and serine residues in TM5 but no direct interaction with TM1 (Figure 16A). Docked ligands were unable to interact with TM1 due to the longer distance between TM1 and TM5 present in β_2 adrenoceptor based models, and due to the occluded space by the residues of TM2 and TM7.

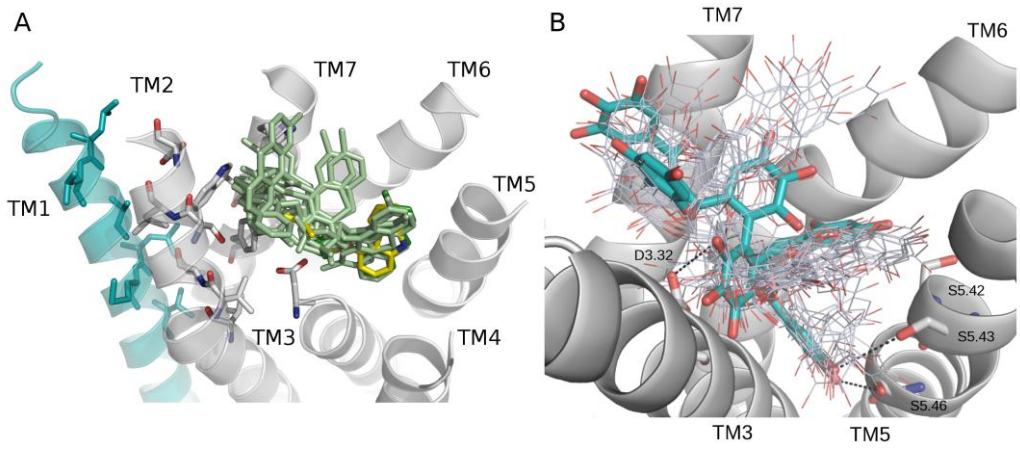
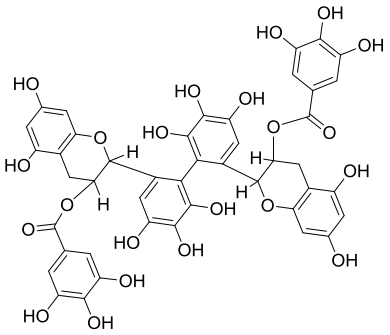


Figure 16 Docking results on α_{2A}-adrenoceptors. On the left figure Docked antagonist (green) do not interact directly with TM1 (in blue). In yellow carazolol, crystallized ligand in β_2-adrenoceptor was used as reference to select docking poses. On the right figure The best ranked docking pose (in blue), interactions with S3.32, S5.43 and S5.46 are marked.

In Publication IV, a strong inhibitor, non-amine, non-protonable compound 2',2'-bisepigallocatechin digallate was found, containging a catecholic group (also present in endogenous catecholamines) (Table 7). Non-charged ligands are unknown to date for α_2-arenoceptors. Docking studies showed that 2',2'-bisepigallocatechin digallate could fit in the orthosteric site and interact with D3.32, S5.42 S5.43 and S5.46 thus, suggesting a direct interaction with the receptor binding site (Figure 16B).

Table 7 Binding affinity of 2',2'-bisepigallocatechin digallate.

Affinity for 2',2'-bisepigallocatechin digallate

	α_{2B}	**α_{2A}**	**α_{2C}**
Structure	**K_i, nM (95% CI)**	**K_i, nM (95% CI)**	**K_i, nM (95% CI)**
	91 (46-176)	14 (6-29)	18 (7-45)

6. Additional unpublished results

A brief SAR analysis was included in Publication II, and the project was continued in order to gain additional insights of the SAR of ABCC2 modulators and to explore how the found inhibitors could interact with the binding cavity in ABCC2. Some of the unpublished results from these experiments follow.

Additional concentration-response curves of 118 compounds were measured using the vesicular transport assay CDCF assay, as used in Publication I and Publication II (presented in section 4.2.1). The analysis was restricted to the CDCF assay based on the findings in Publication II, a good agreement between the results obtained from the EG and CDCF assay and constantly a more robust data producible with CDCF assay. These additional compounds were selected from the University of Pittsburgh, Center for Chemical Methodologies and Library, the same library used to select the compounds used in Publication II.

In these unpublished results, my co-researchers and I identified 16 compounds from this test set with an IC_{50} lower than 30µM. More than half of the tested compounds (72 of 118) belong to one of the four scaffolds presented in Publication II, while the remaining 46 compounds share a new benzenesulfonamide scaffold. Here, the preliminary SAR results for 4-phenyl-3,4-tetrahydropyrimidin-2-one analogues are presented in Table 8.

Within this subscaffold (Figure 17), all molecules have butyric acid in position R_1 and a methyl group in R_4. The variation in the R_2 and R_3 groups are shown in Table 8, a total of 23 molecules of which three are inactive, seven are weak inhibitors, and 13 are inhibitors.

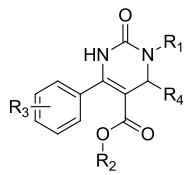
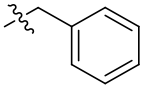
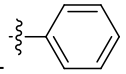


Figure 17 The Markush representation of Scaffold 2.

Table 8 Structure activity relationships of scaffold 2B. Here are presented the variation of R_2 and R_3 substituents. In *bold* font the compound code. In *normal font* IC_{50} (95% confidence interval). Inhibitor, weak inhibitor and inactive classes were assigned when IC_{50} was not possible to calculate. Assignment of classes was made on at least three concentration points, inhibitors have predicated IC_{50} lower than 100µM and weak inhibitor between 100 and 400µM.

-R_3	**-R_2**			
		-CH_2CH_3	-CH_2CBr_3	-CH_3
4-Cl	**2r** 47 (31-72)			**2l** inactive
4-Br	**2ag** 51 (31-83)		**2at** inhibitor	
4-NO_2	**2ad** weak inhibitor			
4-OCH_3	**2t** 135 (66-278)			
3-OCH_3	**2v** 97(72-132)			
2-Cl	**2s** 58 (43-79)			
3-NO_2		**2aj** inactive	**2ar** 25 (16-37)	
2-Cl; 3-Cl	**2au** 15 (13-17)			
2-Cl; 4-Cl	**2ah** 140 (86-227)			
-H	**2ab** inactive	**2i** 85 (63-114)	**2as** 31 (26-38)	
2-NO_2; 4,3 				**2u** 57 (37-87)
4,3 	**2ae** 79 (68-92)	**2j** 71 (47-107)		
2,3 				**2k** weak inhibitor
2,3  ; 5,4 		**2n** 31 (26-37)		
4,3 	**2ac** weak inhibitor			
4- 				**2m** weak inhibitor

Within this group of molecules, the strongest inhibitor showed an IC_{50} of 15µM (**2au**). Some indications on position selectivity for the substitution is shown to preference the ortho-meta substitution compared to ortho-para one, as **2au** has ten times higher affinity then **2ah**. Halogen substitution as in **2ar** and **2as** lead to more active compounds compared to aliphatic hydrophobic substitutions. Additionally, the three molecules (**2j**, **2u** and **2ae)** with 1,3-oxolanein modification at R_3 have an IC_{50} lower than 80µM, indicating that this substituent could be important for inhibitory activity.

Additionally, a pharmacophore with shared features was built in order to illustrate the spatial arrangements of features. The identified pharmacophore for this scaffold showed two hydrophobic features and two hydrogen bond acceptor features shared between all active molecules (Figure 18).

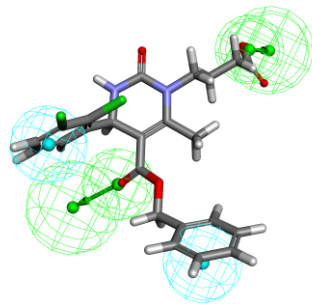


Figure 18 Pharmacophore of Scaffold 2B. Hydrophobic feature (blue), Hydrogen bond donor feature (green), compound **2au** is represented as sticks.

In order to suggest further molecular interactions at the binding site, the homology model of ABCC2 (Figure 19) was built. The mouse mdr1 was selected as the template for protein modelling (Li et al. 2014). The alignment (Figure 20) was obtained from a multiple alignment aligning in which both TMDs and NBDs were aligned independently. There are uncertainties in the alignment of several helices and, therefore, the model is useful only to get an impression of the possible charge and the size of the binding pocket.

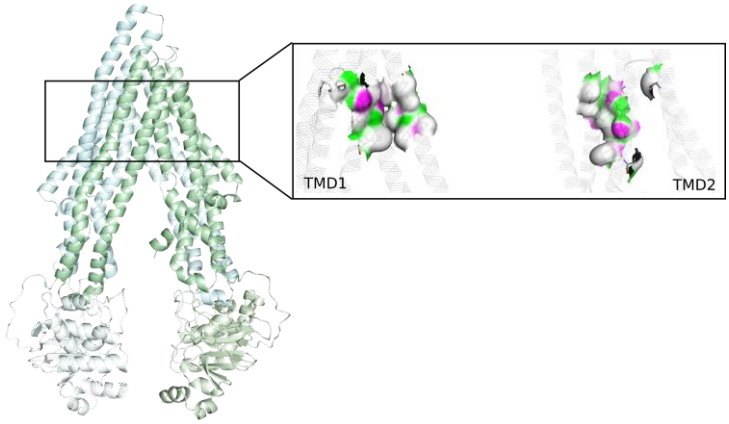


Figure 19 Homology model of ABCC2, with identified the proposed binding site. TMD1 and NBD1 in blue (dark and light); TMD2 and NBD2 in green dark and light respectively. Box identifies the binding site, surface is coloured based on hydrogen bond donor (pink) and hydrogen bond acceptor (green) areas.

The comparison of the pharmacophore model with the predicted binding cavity in the homology model of ABCC2 should be useful in suggesting the amino acid counterparts of interactions that could be later tested using site directed mutagenesis.

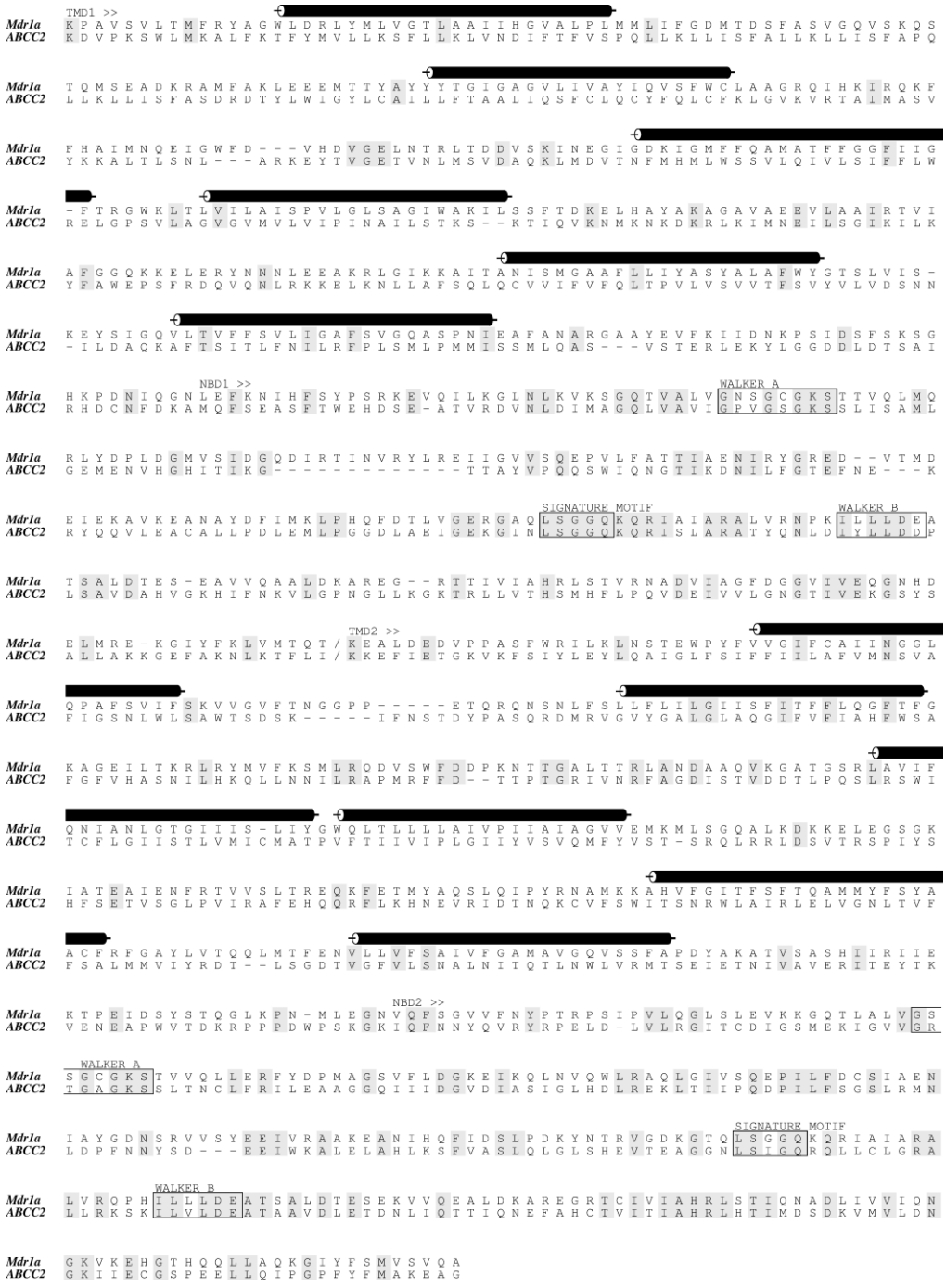


Figure 20 Sequence alignment of ABCC2 and mdr1a. The Predicted secondary structure of ABCC2 based on the mouse mdr1 crystal structure (pdb code 4M2S).

7. Discussion

❖ Identify novel compounds using screening approaches

It is unavoidable that screening, and especially High Throughput Screening (HTS) programs, include false negatives and false positives among the real hits. False negatives normally occur if the assay is not sensitive enough. In addition, the compounds on the plate may have degraded. Poor solubility and resulting precipitation may decrease the actual tested concentration, leading to a decreased signal, or in some cases to false negatives. In a screening campaign, it is usually considered acceptable to "lose" some hits. On the other hand, when a screening approach is used to establish a chemical profile, then it is important that all data points are reliable.

False positive hits are compounds that chemically react to the protein but do not have any biological activity. Hits can be validated, and false positive eliminated, using orthogonal assays. False positives may occur for several reasons; identifying a "false hit", is described in Baell & Holloway 2010. Compounds can interfere as aggregates altering the assay itself or can absorb or can emit at the detected wavelengths altering the detection. Impurities deriving from the synthesis route, like metals that can react with an oxygen singlet or alter the redox cycle producing reactive oxygen species, can directly inhibit the protein activity. EDTA or other chelating agents can sequester important co-factors like metal ions, directly altering the proteins function. Other examples of compounds that could cause false positive hits are sticky compounds that bind non-specifically to the protein, or molecules that covalently bind to the protein altering the function without binding directly to the binding site (Baell & Holloway 2010).

Frequent hitters are compounds that recursively appear as hits in all types of assay, merely because they interfere with the assay itself. It has been strongly suggested to eliminate such compounds both from screening libraries and from any type of SAR analysis (Baell et al. 2013). To eliminate the frequent hitters, the Pan Assay Interference Compounds (PAINS) has been developed, filtering out the compounds with a substructure that has been found in frequent hitters (Baell & Holloway 2010).

In Publication I, polyphenols compounds such as quercertin can precipitate in the assay conditions thus leading to false positive result (Pohjala & Tammela 2012). Quercertin was identified to be an inhibitor of all three probes, this suggests it to either be a real inhibitor or a false positive hit interfering with all three assays.

In Publication II, some inactive compounds were selected for retesting to limit the false negative hits. Several potential frequent hitters can be identified, for example scaffold 1, the tetra-hydro-quinoline. According to the PAINS, tetrahydroquinoline scaffolds displays a 135% enrichment of hits per assay in bioassay databases, and this could interfere with the assay or the detection techniques. Such a scaffold could interfere photometrically with the CDCF assay due to intrinsic fluorescence or quenching effects thus altering assay detection/results. This can be taken into account determining the fluorescence, or quenching effect or using a non-fluorescent probe such as EG. In the specifics, scaffold 1 has showed highly consistent inhibition profiles between CDCF and, EG (a non fluorescent assay) confirming the activity of such scaffold.

A typical HTS hit rate is between 0.5% and 5.0%, depending essentially on the druggability of the target protein and on the selected threshold to consider a compound to be active, which in turn depends on the target and on the aims of the project. Too high threshold will select too many molecules for further evaluation; too low threshold will limit the hits to a very small selection of highly active compounds.

in Publication IV, the hit limit was set to 50% inhibition to select only strong binders at a tested concentration of 10μM. The resulting hit rate was 0.98%, and the 176 molecules identified as hits were then re-tested in a single-concentration assay (1μM). The aim of the project was to identify high affinity (low K_i) α_2-aderenoceptor modulators and subtype selective molecules. Many active compounds already were known.

Additionally, 2',2'-bisepigallocatechin digallate was identified as a new α_2-adrenoceptors inhibitor with nM affinities, this compound has been later mentioned to be highly promiscuous, possibly identifying it as a false positive hit.

- **Specific mode of action and relevance to the *in-vitro* methodologies**

Publication I and Publication II, VT-assays are based on active, ATP-dependent, probe transport. The active probe transport is measured by subtracting the uptake into the vesicle in the absence of ATP (mainly passive diffusion) from the active transport measured in the presence of ATP. Passive probe transport is detected either without ATP or by adding an non-hydrolysable ATP, such as AMP-PNP, which allows the interactions of the NBD but not the hydrolysis (Pratt et al. 2006). The comparison of both assay setup did not show significant difference (data not shown), thus in the assays used in Publication I and Publication II only an assay buffer was used as control.

Human ABCC2 transporters were overexpressed in Sf9 insect cells after a baculovirus transfection. Insect cells have been selected because of their high expression levels and low cost. Background activity, due to endogenous ATP transporters, can be detected and require control measurements. In Publication I, vesicles from β-galactosidase transfected cells were used as a control to validate the ABCC2-ATP dependent transport of the three probes.

The use of stable cell lines (such as Sf9 insect cells) allows researchers to generate large batches that can be prepared and cryopreserved. Transporter activity is known to vary from batch-to-batch, thus the usage of larger batches can decrease the batch to batch variation. Additionally, an advantage of using the VT-assay in respect to cell based systems is the possibility to preload buffers and substrates, making it more high-throughput then a cell based assay.

Membranes obtained from Sf9 insect cells differ from mammalian cells, one major difference being the much lower concentration of cholesterol in insect cell membranes (Kodan et al. 2009; Marheineke et al. 1998). For some ABC transporters, e.g. cholesterol it is usually added in ABCG2 expressing Sf9 vesicles. (Pal et al. 2007). Similarly, ABCC2-transport could be affected by cholesterol. LTC4 mediated transport has respectively K_m values that varies between 1μM (in MDCKII membrane) to 0.690μM (Sf9 cells). Nevertheless, the effect of membrane cholesterol has not been clearly studied (Cui et al. 1999; Heredi-Szabo et al. 2008; Bakos et al. 2000). In addition, transporters expressed in Sf9 cells tend to be less glycosylated and this can alter the transport kinetics. However, this did not seem to effect ABCC2-mediated transport (Bakos E et al. 1996).

Moreover, it is important to consider the effect of the co-substrates and co-factors, such as GSH or Mg^{2+}, which can alter the kinetic parameters. The concentration of co-substrates and co-factors can be a limitating factor for transport, for example Mg^{2+} is important for the ATPase activity. Some evidence suggests that glutathione is often added to the assay reaction since some molecules need it to be transported by ABCC2 (Paulusma et al. 1999). This addition is still strongly debated (Borst et al. 2006). In Publication I and Publication II, glutathione was added; as it was not possible to rule out if for some of the tested molecules glutathione would be an important co-factor for modulation.

For inhibition studies, like in Publication I and Publication II, the probe substrate was shown to have a central role in the evaluation of inhibitors. Optimal probe compounds should be characterized by active transport, low apparent permeability, and low nonspecific binding (to vesicles, filters, and assay plate). The three probes used in these

studies have been recommended as the preferred probes in ABCC2 VT-assays (Hillgren et al. 2013).

Similar permeability restrictions apply even for modulators, as very lipophilic compounds, in addition to non-specific binding, can partition with the membrane, disrupting the structure and making them more permeable (leaky), thus altering the kinetic measurements. To limit the passive permeability, extensive membrane partitioning and unspecific binding to the membrane, it has been recommended to stop the reaction with ice-cold buffer and quickly filter the excess buffer away.

- **Probe-dependent transport in ABCC2-mediated transport**

In Publication I and Publication II, the reason behind the probe-dependent modulation is not known. One hypothesis is due to the different transport mechanisms of the two probe substrates (EG and CDCF). CDCF transport can be described by the classic Michaelis-Menten curve, identifying one single binding site. EG transport instead displays a sigmoidal curve that indicates two possible co-operative binding sites (Zelcer et al. 2003; Bodó et al. 2003).

- **TM1 involvement in antagonist binding on α_{2A}-adrenoceptors**

In Publication III, TM1 is the least conserved transmembrane helix of the seven helices in α_2-adrenoceptors. It has previously been shown that other GPCRs involve TM1 in binding: for example in H1 receptors, the residues 1.35 and 1.39 and in rhodopsin the residues 1.49 and 1.50 interact the ligand (Strasser et al. 2008; Shi et al. 2001). In Publication III, it was shown that TM1 influences the binding affinities of three large antagonists. Molecular docking showed that these antagonists most likely cannot bind simultaneously in the orthosteric site and interact with TM1. Therefore, an indirect effect was proposed which could involve the receptor over all transmembrane packing and dynamic movement of the receptor.

Additional caution should always be observed when analysing results obtained from chimera experiments. Mutated receptors are not biologically and physiologically relevant. Swaps in TM1 can affect the overall packing of the TM helix and the shape of the binding pocket, affecting the affinity of the ligand but without a direct contact. This study has been continued by creating half α_{2A}-adrenoceptors and α_{2C}-adrenoceptors chimeras showing that the half C-terminal domain of α_{2C}-adrenoceptors is important for low receptor expression and drug selectivity, highlighting (extracellular loop2, intracellular loop 1 and C-terminal) as possible regions involved in selectivity (Jahnsen & Uhlén 2013).

❖ Homology models used to understand the ligand biological activity

The usefulness of homology models depends on their quality. An important factor when evaluating homology models is the sequence identity between the target and the template and the correctness of the alignment.

It has been estimated that if the sequence identity between the template and the target is higher than 50%, the calculated rmsd (from the experimental structure) is of about 1Å; while for two sequences that have a sequence identity of 30–50%, the overall structural similarity is estimated to be approximately 80% with an rmsd of 2-3Å (with the majority of the errors in the loop prediction). When the sequence identity drops to 20-30%, the structural similarity is predicted to be only 55%, which can decrease to 20% with a lower sequence identity (Kryshtafovych et al. 2005). As a reminder, homology models are only models and cannot represent the reality better than the template itself (low resolution structure, low resolution model). In addition, models do not represent the full dynamic picture of the protein flexibility and the conformational changes that occur upon protein-ligand binding. Additional inaccuracies will occur due to the natural evolutionary divergence between the target and the template.

Homology models have been built to better understand the structural determinants in the ligand binding of α_2-adrenoceptors. Initially, models were based on bacteriorhodopsin and frog rhodopsin. Using these models, it was possible to identify only the general seven transmembrane helices and the general localisation of binding cavity residues (Mizobe et al. 1996; Salminen et al. 1999). In 2000, when the bovine rhodopsin finally was crystalized with 2.8 Å resolution, it was possible to increase the resolution of the previous models (Rosenbaum et al. 2007). In the last 5-10 years, 28 unique GPCR have been crystallized, but the α_2-adrenoceptor is still lacking (Vroling 2014).

In Publication III and Publication IV, the homology model of α_2-adrenoceptors subtypes is to be considered reliable and predictive. The models were built on the alignment with the β_2-adrenoceptor where a higher level of sequence identity is present in the TM regions (the calculated sequence identity varies between helix between 37-43%).This has been illustrated in the GPCR dock competitions (2008, 2010, 2013) that a sequence identity of 35-40% is the empirical cut-off for reliable homology models of GPCRs (Kufareva et al. 2014) .

In the unpublished data, it was not possible to achieve a high sequence identity for ABCC2 – template (mdr1) alignment, since the overall sequence identity was 18% (with 30% in the NBD and only 10% TMD). The mouse mdr1 was selected as template

for the ABCC2 model, since it is one of the crystal structures with the highest sequence identity and due to the presence of a co-crystallized inhibitor cyclic-tris-(S) valineselenazole (QZ59-SSS). ABCB10 showed a bit higher sequence identity with ABCC2 (22% versus 18% with mdr1) but it has been crystallized in the closed conformation and does not have a co-crystallized ligand.

Due to the low sequence identity between the target and the template, uncertainties are present in the predicted model. Therefore it is not possible to define the exact position of the amino acids in the model and, as a consequence, the interaction between ligands and transporter. Even if the predictive model presents some uncertainties, it can describe in a general (non-specific) way the characteristics of the ABCC2 binding cavity. Additional uncertainties are the model come from the TMD0 that is not present in the template but present in ABCC2 and, therefore, cannot be reliably modelled.

8. Conclusions and perspectives

Many molecules are known to interact with the ABCC2 transporter, and some of them are transported. As a future prospective, it would be interesting to define which of these modulators are actually transported (the current VT-assay does not detect actual transport). In addition, it would be interesting to increase the number of concentration points in the determination of the IC_{50}, to reduce the uncertainty in the results, and to lower the highest concentration point (400µM) reducing the risk of precipitation associated with poorly soluble compounds. Controls with mock cells could be added for each tested compound to take into account the possible transport/existence of insect ABC transporters.

In Publication I and Publication II, probe-dependent modulation was studied showing that some modulators are probe-dependent. An hypothesis that can explain this probe-dependence is the different transport mechanisms of the probes. Several studies have proposed that ABCC2 does not have one single well defined binding site, but possibly two sites that can cooperate in the transport of substrates. Probe selective modulators could be helpful in understanding the mechanism of transport of the probes and the reason behind such different modulatory effects. Another explanation that is not ruled out in the current settings is an artefact in measurements due to intrinsic fluorescence or on CDCF fluorescence quenching.

In Publication II, as a results of the comparison the two assays, it was shown that CDCF assay is more robust then the EG assay. Identifying the CDCF assay is more attractive for future screening projects, thanks even to the lower cost of fluorescent assays (CDCF assay) respective of the radiolabeled ones (EG and LTC_4 assay).

There is a need for α_2-adrenoceptors subtype specific molecules that can be used in the treatment of high blood pressure, in the alleviation of withdrawal syntomes by inhibiting the processing of sensory information, and as anaesthetic with a safer therapeutic profile compared to the drugs currently used that are non-selective. The known α_2-adrenoceptors subtype-selective drugs have a K_i in the order of low nM. The compounds that have been identified with miniaturized HTS in Publication IV have shown to have similar K_i on the low nM range and, thus, are ideal hits for further drug discovery optimisation.

Challenges in designing subtype specific molecules are expected due to high structural similarity of the subtypes. Subtype specificity is still hard to address, as receptors share

a very similar binding cavity, suggesting that ligands are accommodated similarity. TM1 was shown to be involved in subtype determination of three antagonists in Publication III. However, docking results showed that ligands which bind to TM3 and TM5 do not have direct contact with TM1.

Since the 3D structure of α_2-adrenoceptors is not available, molecular models have been used to define the binding site, nevertheless, having an intrinsic uncertainty. The α_2-adrenoceptors binding site has been defined in Publication III based on the β_2 adrenoceptors. Recently crystallized dopamine D_3 receptor would be an interesting template to base a new homology mode of α_2-adrenoceptors, having higher sequence identity.

An additional value of Publication II and IV is the collection of two screening studies on ABCC2 and α_2-adrenoceptors subtypes, measured under the same conditions and laboratory and, therefore, considered a perfect data set for further computational analysis. In the specifics of Publication II, it is the first large screening campaign performed with two probes. The data could be further utilized to predict transporter-drug interactions or to develop SAR model for ABCC2 inhibitors.

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