Molecular Basis to human P-glycoprotein reversion

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Abstract

ABC (ATP-binding cassette) transporters are involved in translocate a wide spectrum of molecules across the lipid bilayer and some of them are associated with various diseases. They also have an important role in multidrug resistance (MDR) in cancer, which has been a major obstacle in cancer chemotherapy and in the treatment of leishmaniasis. While diverse transporters may confer MDR phenotype in bacteria, in human it is mainly achieved by five ABC proteins, among them P-glycoprotein/(ABCB1). To understand the structural basis of P-gp inhibitory activity, to determine the ligand binding sites within P-gp and to guide the design of more potent P-gp inhibitors, we performed *i)* a 3D-QSAR model using CoMSIA on a set of sesquiterpenes, *ii)* molecular docking simulations of various compounds in a homology model of LMDR1, a P-gp-like transporter belonging to the *Leishmania* ABC family and *iii)* and a complete study of sesquiterpenes interaction with human P-gp.

Resumen

Los transportadores ABC (ATP binding cassette), encargados de transportar un amplio espectro de moléculas a través de la bicapa lipídica, pueden estar asociados con diversas enfermedades. Juegan un papel importante en la multirresistencia a fármacos (MDR), obstáculo importante en la quimioterapia del cáncer y en el tratamiento de leishmaniasis. En bacterias varios transportadores pueden conferir el fenotipo MDR, en humanos son principalmente cinco, entre ellos la glicoproteína P/(ABCB1). Para comprender la base estructural de la actividad inhibidora de P-gp, determinar los sitios de unión de los ligandos a P-gp y diseñar inhibidores de P-gp más potentes, se realizó *i)* un modelo 3D-QSAR usando CoMSIA en un conjunto de sesquiterpenos, *ii)* simulaciones de acoplamiento molecular de varios compuestos en un modelo de homología de LMDR1, transportador de la P-gp perteneciente a la familia ABC de *Leishmania* y *iii)* un estudio completo de interacción entre sesquiterpenos y la P-gp humana.

Preface

ABC (ATP-binding cassette) transporters embrace one the largest families of transmembrane proteins, which is found in almost all cells, from bacteria to mammals. They are primary transporters that use the power of ATP molecules to translocate a wide spectrum of molecules across the lipid bilayer, against the electrochemical gradient; covering from small organic and inorganic compounds, to a large organic molecules, such as lipids, peptides and polysaccharides. The ABC superfamily is divided in seven subfamilies from ABCA to ABCBG. To date, there are 49 ABC transporters identified in humans, and some of them are associated with genetic diseases, such as bleeding disorder, eye disorders, lipid transport and liver diseases, cystic fibrosis, and immune deficiency and multidrug resistance in cancer. Multidrug resistance (MDR) refers to the phenomenon whereby cells develop resistance to a number of drugs with very diverse molecular structures and mechanisms of action.

While diverse transporters may confer MDR phenotype in bacteria, in human it is mainly achieved by five ABC proteins: P-glycoprotein/MDR1(ABCB1), MDR2(ABCB4), MRP1(ABCC1), MRP2(ABCC2), and BCRP1(ABCG2).
P-gp is an ABC exporter capable to transport a wide spectrum of structurally unrelated compounds across the cytoplasmic membrane.

The ability to interact with such a large number of structurally unrelated compounds is in controversy with the classical lock-key model for enzymes. The capability of MDR proteins to bind multiple substrates, named “polyspecificity”, or “promiscuity”, is achieved thanks to large and flexible binding pockets to which one, or multiple ligands at the same time, can bind within the same binding site. These characteristics made the development of P-gp inhibitors a great challenge, as demonstrated by the low specific inhibitor available and their scarce selectivity.
In the last years a new group of ligands, sesquiterpenes, have been show to actively and selectively inhibit P-gp. Sesquiterpenes are natural compounds extracted from essential oils of the *Celastraceae* plants and have attracted considerable attention from synthetic organic chemists and pharmacologists due to their complex

structures and wide range of biological properties. The sesquiterpenes MDR reversion ability have been ascribed to their specific interaction with P-glycoprotein, as they poorly interact with other human ABC transporters homologs that cause MDR, such as MRP1, MRP2 and BCRP.

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1. INTRODUCTION

Chapter 1.

INTRODUCTION

1.1 ABC Transporters

1.1.1 General overview

ABC (ATP-binding cassette) transporters embrace one the largest families of transmembrane proteins, which is found in almost all cells, from bacteria to mammals [1]. They are primary transporters that use the power of ATP molecules to translocate a wide spectrum of molecules across the lipid bilayer, against the electrochemical gradient; covering from small organic and inorganic compounds, to a large organic molecules, such as lipids, peptides and polysaccharides. Transport of solutes across the cell membrane is indispensable for living beings, since it permits to maintain an net off chemical equilibrium state. ABC transporters act as exporters in eukaryotes, excreting solutes from the cytoplasm to the outside of the cell, or into an intracellular compartment (endoplasmic reticulum, Golgi apparatus, mitochondria, and peroxisome). As well, ABC transporters may behave as importers of nutrients and excretory pumps of toxins in prokariotes[2].

The ABC superfamily is divided in seven subfamilies from ABCA to ABCBG [3]. Genes that encode ABC transporters are widely dispersed among the genome and show a high degree of amino acid sequence identity among eukaryotes [4]. To date, there are 49 ABC transporters identified in humans [5], and some of them are associated with genetic diseases, such as bleeding disorder[6], eye disorders [7], lipid transport [8] and liver diseases [9], cystic fibrosis[10, 11], and immune deficiency and multidrug resistance in cancer[12, 13].

An ABC transporter functional unit is form by four domains: two transmembrane domains (TMD) embedded in the membrane, which are the responsible of binding and translocation of solutes through

the membrane, and two nucleotide-binding domains (NBD) located in the cytoplasm, which hydrolyses two ATP molecules and power substrate transport in the TMDs [14] [14] [15]. Additional regulatory domains can be fused to the TMDs as well as high affinity binding proteins in prokaryotic importers, which deliver solutes from the periplasm to the TMDs[16].

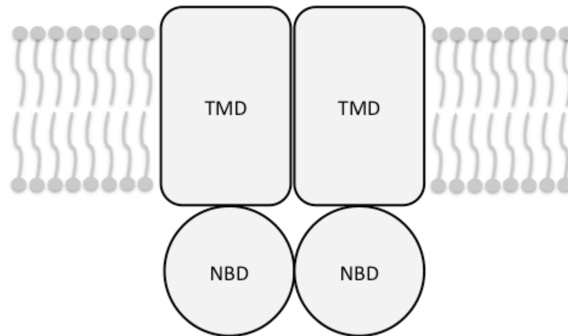

Figure 1. Schematic representation of the four minimal domain units of a functional ABC transporter, composed by two transmembrane domains (TMD) and two nucleotide-binding domains (NBD).

These four minimal domains, two TMDs and two NBDs, can be fused in a single aminoacid chain or assembled by different monomers expressed in two, three or four different polypeptide chains [17]. In bacteria, the functional unit can be composed by a single polypeptide chain containing the four domains (full transporter); or by two equal or distinct chains forming a dimer (half transporters), each monomer containing one TMD and one NBD; or by a combination of single/fused NBDs and single/fused TMDs. ABC transporters of eukaryote are mainly full transporters, with two TMDs and two NBDs encoded in a single gene, or half transporters formed by two equal or distinct monomers, of one TMD and one NBD each one, forming homo or heterodimers [18, 19].

TMDs are fold by diverse packed α-helices, spanning the membrane such a way that shape the pore for solute transport across the membrane [20-24]. Usually, ABC transporters have 6 transmembrane helices (TMH) at each TMD, but the number can vary from 6-10, for a total of 12-20 TMHs for a complete

transporter[15]. At the initial state of the ABC transport cycle, the TMDs are open to one side of the membrane in a high affinity conformation for substrates; exporters are open to the cytoplasm and importers to the outside/periplasm. Major movements of the TMDs and rearrangement of their helices, powered by NBDs, causes the opening at the other site of the membrane and subsequent release of substrate. Transmembrane helices extend into cytoplasm several Å beyond the membrane to reach the NBDs. TMDs are swapped in such a symmetrical way that each TMD is coupled to both NBDs by different pairs of helices.

Therefore, TMD-NBD coupling transmits the energy from ATP molecules to the TMDs, and triggers the structural changes in the TMHs during the transport cycle [25]. Despite all ABC transporters share the same TMD topology, there is no conservation between the different TMDs. This is likely due because of the different substrate selectivity of ABC proteins. The different substrate selectivity of ABC transporters lay on the TMDs, since solutes must bind with the residues of the pore formed by TMD in order to be translocated through the pore. On the contrary, NDBs aminoacid sequences present a high level of conservation, they share some conserved motifs common to other nucleotide binding proteins (Walker A, and Walker B motifs), and some other characteristic of ABC transporters family (ABC-signature, Q-loop, and H-loop).

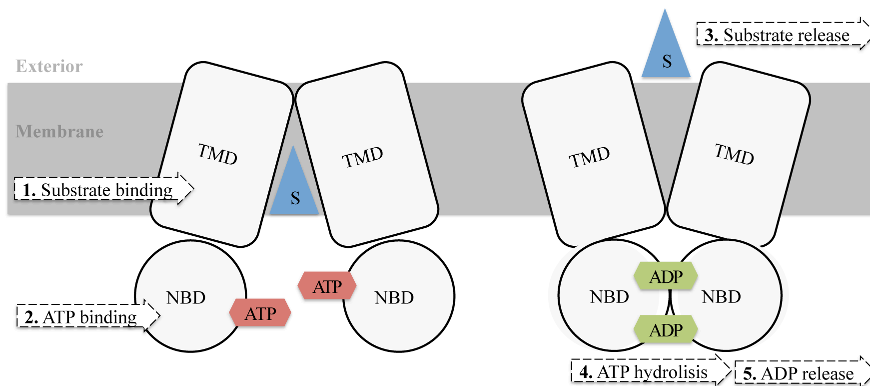

Figure 2. Representation of the transport cycle of a typical ABC exporter. Substrate binding at the transmembrane domains (TMD) triggers ATP binding and nucleotide binding domain (NBD) dimerization, which produces two major changes: i) movements in the TMDs that release substrate out of the cell, and ii)

ATP hydrolysis and ADP release. Finally, NBDs dissociation forces transporter to the initial conformation to start a new cycle.

Although there are still some features of the catalytic cycle of ABC transporters that remain unknown [26], it is generally assumed that at the beginning of cycle the NBDs are separated away in a low affinity state for ATP and that TMDs are open to one side of the membrane in a high affinity for substrate state. Binding of substrates at the TMDs, which are coupled to the NBD, triggers ATP binding and NBDs dimerization[25], which, at the same time, cause large changes in TMDs that lead to the low affinity conformation for substrate, and release of substrate at the opposite side of the membrane. The NBD-NBD interface compose the catalytic site, by packing together Walker A, Walker B, and the Q- and H-loop patterns of each monomer. At this step of the cycle, with the two ATP molecules sandwiched between the NBDs, is when the transition state occurs, which derives in ATP hydrolysis and ADP+P_i release. Therefore, ATP catalysis triggers NBD-NBD dissociation and resets the transporter to the initial state of the cycle[27, 28].

1.1.2 Multidrug resistance and polyespecificity

Multidrug resistance (MDR) refers to the phenomenon whereby cells develop resistance to a number of drugs with very diverse molecular structures and mechanisms of action. The MDR phenotype typically occurs when drugs are administrated in clinical treatments for infections and cancer. Pathogens and tumor cells have the intrinsic, or acquired under pharmacological treatment pressure, ability to forbear drugs. This is one of the major causes of chemotherapy failures; such as in bacterial infections, malaria, leishmaniosis and cancer. Living cells can develop diverse strategies to survive to drugs that have never been exposed before.

One of the most common mechanisms of MDR is by decreasing membrane permeability of drugs by overexpressing different classes of membrane transporters [29]. ABC transporters are one of the main families of proteins responsible for decreasing intracellular accumulation of drugs within the cells[13, 30]. These transporters

translocate a wide variety of substrates across the membranes, and when mutated or overexpressed they are capable of making cells insensible to drugs. Hence, clinical dosages become insufficient to achieve optimum drug plasmatic concentrations and as consequence, higher doses lead to adverse side effects and unacceptable toxicity levels for patients. While diverse transporters may confer MDR phenotype in bacteria, in human it is mainly achieved by five ABC proteins: P-glycoprotein/MDR1(ABCB1), MDR2(ABCB4), MRP1(ABCC1), MRP2(ABCC2), and BCRP1(ABCG2).

The ability to interact with such a large number of structurally unrelated compounds is controversy with the classical lock-key model for enzymes. The capability of MDR proteins to bind multiple substrates, named "polyspecificity", or "promiscuity", is achieved thanks to large and flexible binding pockets to which one, or multiple ligands at the same time, can bind within the same binding site. Some good examples of polyspecific binding are displayed with the crystal structures of the soluble transcript factor QacR from *S.aureus* bound with six different substrates[31] and with two substrates bound simultaneously [32] [33]; and BmrR from *B.subtilis* [34] [35], the AcrB multidrug efflux Pump from *E.coli* with four substrates bound[36], and the mouse P-gp crystal structures with two [37] and four different substrates bound[24]. Each of these structures display different ligands, individually or simultaneously, bound at the same cavity, where each ligand is able to bind by using a different subset of aminoacid residues. The binding pockets of these structures are shown as a large and flexible cavities shaped by multiple hydrophobic and aromatic residues, thus revealing an induced fit mechanism by which protein and ligand adapt to each other, mostly by stacking and van der Waals contacts.

1.2. P-glycoprotein

1.2.1 Tissue distribution and physiological role

P-glycoprotein (P-gp, MDR1, ABCB1) is a glycosylated membrane protein of 170kDa, and is the first discovered ABC transporter capable to confer MDR [38, 39]. Ling and Juliano reported in 1976 that surface membrane permeability of Chinese hamster ovary (CHO) cells was modulated by a membrane glycoprotein, naming it P-glycoprotein (P for permeability). Since then, many ABC transporters have been identified, and our knowledge of the mechanism of action, structure, and modulation of this family of proteins has been enriched during the last years.

P-gp is an ABC exporter capable to transport a wide spectrum of structurally unrelated compounds across the cytoplasmic membrane; such as Colchicine, Rhodamine 123, Hoescht 33342, vinca alkaloyds, anthracyclines, epipodophyllotoxins, Paclitaxel, Colchicine, cyclic and linear peptides, HIV protease inhibitors, and many other compounds [40].

P-gp is expressed in most mammals tissues at low levels, while is highly expressed in epithelia and endothelia of excretory organs [41] such as pancreas, liver (canalicular membrane), gut (apical membrane), and kidney (apical membrane of epithelial cells of proximal tubule). Moreover P-gp is highly expressed in the blood-brain barrier [42] (BBB) (apical membrane of endothelial cells), blood-inner ear barrier [43], testis (endothelial cells of capillary), at the luminal surface of secretory epithelial cells in the pregnant endometrium [44], placenta[45], and hematopoetic cells.

Additionally, P-gp is also expressed in other subcellular membranes in cancer and MDP phenotype cells, such as mithocondrial [46] [47], Golgi apparatus [48], and nuclear membranes [49].

Therefore, P-gp plays a major role conferring broad-spectrum protection against xenobiotics and excreting metabolites out into the

urine, bile, and lumen of intestines. Subsequently, P-gp interferes absorption, distribution, metabolism, excretion, and toxicology (ADMET) properties of most drugs [40] [50], and confers a MDR phenotype to cancer cells that develop cross-resistance to multiple drugs [51] [52], resulting in chemotherapy failure in near 90% of metastasizing tumors.

1.2.2 P-glycoprotein inhibitors

In the light of this primary role in drugs ADMET P-gp quickly became a major goal of clinical research [53] and as a consequence many efforts have been made in the last years to develop new P-gp inhibitors. Verapmil, a calcium channel blocker, was the first modulator of P-gp discovered in 1981 [54] and since then, many others drugs have been studied as possible P-gp modulators, like calcium or sodium channel blockers[55] [56] [57], calmodulin antagonists[58, 59], cyclic peptides and macrolide compounds[60], indole alkaloids[61], and multiple flavonoid, steroid antibiotics, surfactants and other m iscellaneous compounds [62]. All these compounds produced disappointing results *in vivo* due to their low P-gp affinity as they caused cross-side effects with other transporters like MRP1, or enzymes like Cytochrome P450, thus altering ADMET properties of drugs. Therefore, these P-gp inhibitors required of high doses up to a toxic levels; and/or caused unexpected side effects[52].

With the aim to find more potent and safe P-gp modulators, the named "second generation P-gp inhibitors" were developed. Chemical modifications of previous P-gp inhibitors derived into more potent inhibitors, such as valsopodar (PSC-833) [63], which showed to be a more specific and safe P-gp inhibitors[52]. Nevertheless, clinical trials showed that they still caused many side effects and altered ADMET properties of drugs. Rational drug design and combinatorial chemistry studies lead to the "third generation inhibitors", which showed highest P-gp affinity (at the nanomolar range) and to be even more specific. Great improvements were made on P-gp inhibition by compounds such as ealcridar (GF120918)[64], zosuquidar (LY335979)[65], biricodar

(VX-710) [66], laniquidar (R1011933) [67], OC144-093 [68], and tariquidar (XR9576) [69]. Again, despite the promising results of this new generation of compounds, they failed undergoing clinical trials as a result of adverse side effects and toxic accumulation of chemotherapeutic drugs.

Therefore, new strategies have arisen for the discovery and improvement of new P-gp inhibitors, "fourth generation P-gp inhibitors". Given the knowledge that some food agents and plant extracts interfere in the bioavailability of several cytotoxic drugs (orange, grapefruit, curcumin, diterpenes, triterpenes, sesquiterpenes, flavonoids, alkaloids, cannabinoids, lipidic and peptide-like compounds, among others) many studies focused on the research of new natural compounds that could be employed as lead compounds of a new class of more specific and safe MDR inhibitors [70, 71].

1.2.3 Translocation mechanism

To date, the exact mechanism by which P-gp binds and expels substrates out of the cells is not completely defined. The early assumption of a classical membrane solute transporter pump, which binds hydrophilic substrates from the cytoplasmic side across the membrane through an internal pore lined by polar residues, didn't seem realistic for P-gp[72, 73]. To date, two translocation models both assuming binding of substrates within the membrane are widely accepted, the "flippase" and "hydrophobic vacuum cleaner" models. One feature shared by many ABC transporters, like P-gp, is the high hydrophobicity of their substrates that can they easily solvate into the membrane and cross it by passive-diffusion[74, 75]. Both translocations models assume binding of substrates from the membrane but they are slightly different regarding the specific site within the membrane where substrates bound. Accordingly, binding site of P-gp has been determined in the TMD, and mouse P-gp crystal structures[24, 37] show two entrances indicating that substrates can access the internal chamber from the lipid membrane.

Due to the high hydrophobicity of P-gp ligands, the "hydrophobic vacuum cleaner" [72, 76] was the first model suggesting substrate

binding within the membrane core followed by excretion of substrates to the aqueous phase out of the membrane.

Besides, many ABC transporters, such as ABCB4 and the P-gp homolog MsbA, behave as flipasses, translocating lipids from the inner leaflet of the lipid bilayer to the outer leaflet. P-gp mediated transport of lipids from the inner to the outer leaflet of the membrane has been also observed, suggesting a flippase activity of P-gp[77, 78].

Both models propose binding of solutes partitioned into the membrane, but while the “flipasse” model indicates binding from the inner leaflet of the membrane and release to either the outer side or out of the cell, the “hydrophobic vacuum cleaner” proposes binding from the core of the membrane and substrate release directly out of the cell. Several experiments of P-gp transport support both of the proposed models [79] [80] [81] [82] [78] [83, 84]. Nevertheless, it is difficult to distinguish between transport of such hydrophobic compounds either to outer leaflet of the membrane and to the aqueous phase given that they would rapidly dissolve into the membrane again.

It is worth to note that binding of substrates solvated into the membrane enhances the polyspecific feature of P-gp and MDR transporters. It is likely that many of the broad spectrum of structure unrelated P-gp substrates perform weak interactions with P-gp, however, lipophilic substrates would be present at higher concentrations inside the lipid bilayer and even having high binding constant (Km) net transport would be favored by accumulation of ligands inside the membrane[85] [86] [87].

1.2.4 Structure

Human P-glycoportein (P-gp/ABCB1) is a full transporter member of the ABCB subfamily composed by a single polypeptide consisting of 1280 amino acids [38], it is divided in two sub-parts, each one composed of one transmembrane domain (TMD) and one nucleotide-binding domain (NBD), linked by an intracellular linker polypeptide chain (~60 amino acids), and it is N-glycosylated in the

first extracellular loop. Sequence analyses indicate that P-gp arised by gene duplication event by fusing two homologous halves [88, 89]. The role of the N-glycosilation at the first extracellular loop is unclear, but it seems to be related with trafficking and stability within the citoplasmatic membrane [90, 91].

One of the most noticeable features of P-gp is its ability to transport a wide spectrum of structurally unrelated compounds across the citoplasmatic membrane,resulting in MDR phenotype [92] [93] [94]. The P-gp binding site is formed by the assembly of the two TMDs within the cytoplasmic membrane, from where substrates can access the internal chamber of the protein by two possible entrances[37]. ATP hydrolysis at the NBDs provides energy to a undergo a major conformational changes, both of them already crystalized.[21, 37] Substrates bind the inward facing conformation, which is closed to the extracellular medium, and are released to the extracellular side after the conformational change to the outward facing conformation [21].

The first insights of P-gp 3D-structure were two electron microscopy low-resolution crystals at 25Å [95] and 10Å [96]. These data indicated that P-gp binding site was formed by and internal chamber shaped by the TMDs, and that different conformational states in the presence/absence of ADP and ATP were possible; thus, supporting the ATP switch model[25]. The crystal structure of the bacterial SAV1866, was the first high resolution (3Å) structure of a human P-gp homolog made available[21]. It was resolved with two ADP molecules bound and displayed an outward facing conformation with the two NBD in close contact; the same protein was later also released with AMP-PNP (an non-hydrolyzable analog of ATP) displaying the same conformation as previous structure[97].

The data obtained by SAV1866 crystal structures shed light over the topology of TMD of ABCB multidrug transporters and allowed the correction of the previous MsbA (bacterial lipid A transporter homolog of P-gp) crystal structures[98]. MsbA from *E.coli* and *V.cholerae*, were solved in an open inward facing conformation at 5.3Å and 5.5Å, respectively. Three structures of MsbA from *S.typhimurium* were solved in the outward facing conformation, as showed in SAV1866 crystal structure, at 3.7Å and 4.5Å with AMP-

PNPbound, and at 4.2Å with ADP+V_i bound. Due to low resolution of the crystals, the authors published the full-atom model of the better-resolved *S.typhimurium* structure with AMP-PNP bound at 3.7Å, and only the Cα trace for the remaining part of the protein.

The first mammalian ABC crystal structure reported was the mouse P-gp in 2009,[37] released in different forms: an apo protein at 3.8Å in an inward facing conformation, similarly to the MsbA from *E.coli*; a cyclo-peptide ligand-bound protein at 4.4Å , and another with two ligands bound simultaneously at 4.35Å. These crystals exhibited a huge internal cavity, of about 6000$Å^3$, within the TMD, accessible from the inner leaflet of membrane by two entrances located between TMH4-TMH6 and TMH10-TMH 12. These crystals revealed how different ligands can bind, individually or simultaneously, within the binding pocket. The internal chamber of the TMDs is filled with hydrophobic and aromatic residues that allow different kinds of interactions with ligands. The mouse P-gp structure conformation confirmed the previously published biochemical data about P-gp binding sites. Indeed simultaneous binding of ligands and the existence of multiple binding sites within P-gp had been extensively reported [99] [100] [101-103] [104] [105] [106] [107] [108] [109] [110] [111] [112], and the mouse crystal structure showed how residues involved in drug binding are located along the large internal cavity formed by the TMDs, allowing polyspecific interactions with multiple unrelated compounds [92] [113].

New structures opened the door to the study P-gp inhibitors by structure-based approaches, due to the high sequence identity of mouse P-gp to the human orthologue (87%), the identification of the different binding sites, and the fact that the inward facing conformation is supposed to represent the high affinity binding state for substrates, opposite to the open outward conformation previously released[25].

Nevertheless, an open debate about whether the inward open state exists in physiological conditions or it was just a crystal artifact raised[114]. Although the structure was consistent with biochemical studies proving that the binding cavity is able to accommodate two ligands simultaneously, some unresolved features still existed. Moreover, the relatively low resolution and the lack of a membrane

environment brought into question the ~30Å distance between NBDs. This large NBD-NBD distance means that P-gp has to undergo huge movements to associate NBDs and hydrolyze ATP, while, both SAV1866 crystals showed NBDs associated in the presence of either ADP or AMP-PNP. Projections structure of P-gp in a membrane environment obtained by electron microscopy[115] (20Å resolution) showed that NBDs were associated under physiological conditions. Besides, ATP is likely to be always bound to the NBDs, since its affinity for P-gp (K_m 0.3-1 mM) is far below the plasmatic concentration of ATP (3-5 mM)[116] thus, a free ATP state of P-gp, as shown in the mouse crystal structure, seems unlikely to exist under physiological conditions. Moreover, the fact that both P-gp structures bound with or without ligands showed the same conformation was in controversy with the induced fit model of P-gp. Additionally, cross-linking studies demonstrate that human P-gp has a high basal activity even when TMDs [117] and NBDs are cross-linked together [118].

These doubts have been dispelled thanks to the numerous P-gp structures in the inward facing conformation released in the last years: *C.elegans* P-gp [119], another mouse P-gp [120], an improved structure of the original mouse P-gp, to which major registry shift corrections were made in several helices[121], as well as a recent new mouse P-gp structure with four different cyclic peptide-like inhibitors bound to [24]. These new Crystal structures showed different separation distances between NBDs, both smaller and larger than the first mouse P-gp.

Furthermore, other crystal structures of ABC transporters have been solved in the last years, such as the human mitochondrial transporter ABCB10[122], the Atm1 from *N.aromaticivorans* [123] and *S.cerevisiae*[124], CmABCB1 from *C.merolae* [125], McjD from *E.coli* [126], TM287/288 heterodimeric exporter from *T.maritima* [127], and the recently published structure of PCAT1 transporter from *C.thermocellum*[128].

The crystal structures released to date confirm the large flexibility of this class of proteins and that they can adopt multiple conformations during the transport cycle. Indeeda part of the outward facing structures open to the extracellular side and with NBDs packed together, various proteins structures with TMDs open

to the cytoplasm exhibit a large range of apertures. For example, P-gps, ABCB1 from *C.merolae*, and the bacterial Atm1 structures show an inward facing conformation with NBD disassociated at different distances; while human ABCB10, PCAT1, and the *T.maritima* transporter offer inward facing conformations with NBD associated. Additionally, FRET of P-gp studies also pointed to different ranges of inter-NBD possible distances [129]; and cross-linking studies showed that P-gp with NBD cross-linked at 20-25Å separations is active too.

Although no crystallographic structure of human P-gp is available to date, and many questions about the physiological conformation of P-gp are still unsolved, the new set of crystal structures offers a large variety of conformations and opens the door to new structure-based studies.

1.3 Sesquiterpenes as P-gp MDR inhibitors

Sesquiterpenes are natural compounds extracted from essential oils of the *Celastraceae* plants. Sesquiterpene polyesters with a dihydro-β-agarofuran skeleton are the most widespread and common group of secondary metabolites isolated from the *Celastraceae* species, and have attracted considerable attention from synthetic organic chemists and pharmacologists due to their complex structures and wide range of biological properties[130] [131]. They have been used since long time ago as insecticide in agriculture [132], and in the last years new pharmacological properties have been reported such as a antiviral [133], inmunosupressor [134], antiinflamatory [135], and multidrug resistance reversion in Leishmania [136] and cancer [137].

Sesquiterpenes polyesters are alkaloid compounds derived from isoprene; they feature a dihydro-β-agarofuran skeleton, which share a tricyclic structure core of 15 carbon atoms. They are characterized by this rigid skeleton with different esterifying substituents that ranges from methyl and hydroxyl groups through acetic and more complex heterocyclic acids, such as benzoic, furoic, or nicotinic acid. The relative position, number, and configuration of these substituents, distinguishes each individual sesquiterpene.

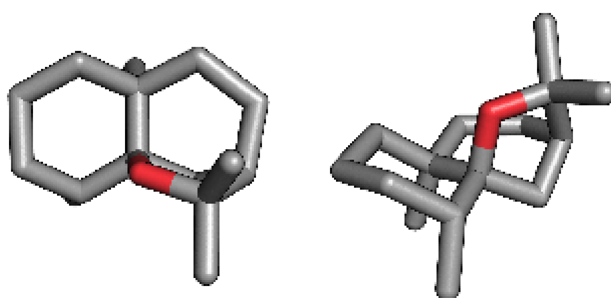

Figure 3. 3D view of the dihydro-β-agarofuran skeleton.

Due to their multidrug resistance reversion ability, a series of different natural and synthetic sesquiterpenes have been studied. Sesquiterpenes have been shown to overcome P-glycoportein

mediated transport of drugs in both Leishmania and tumor cells lines [137] [138] [139, 140].

The sesquiterpenes MDR reversion ability have been ascribed to their specific interaction with P-glycoprotein, as they poorly interact with other human ABC transporters homologs that cause MDR, such as MRP1, MRP2 and BCRP. Moreover, sesquiterpenes have been shown to bind within the TMD of P-glycoprotein, at two different binding sites, without competing for ATP binding at the NBDs. Therefore, sesquiterpenes compete for substrates that bind within the binding pocket of P-gp's TMD, and they appear to be either poorly or not transported. Indeed, due to their high lipophocility, they quickly diffuse into the membrane, thus net transport is difficult to measure.

Biochemical analysis determined sesquiterpenes potency and type of inhibition of P-gp mediated transport. They behave as mixed-competitive inhibitors, capable to stimulate ATPase activity at low concentration, while inhibiting it at higher concentrations. Along with other drugs, sesquiterpenes inhibition studies of P-gp-mediated transport of daunomycin into NIH-3T3 MDR1 cells were carried out, in order to determine the kind of interactions between different pairs of sesquiterpenes and modulators. This experiments revealed different types of inhibition (competitive and non-competitive), and also synergic cooperation of sesquiterpenes with different drugs.

One of the most potent sesquiterpenes (MAMA12/Compound40), which is more potent than the classical inhibitor modulator verapamil, was shown to interact competitively with Cyclosporine A and verapamil, and non-competitively with progesterone and vinblastine. Cooperative inhibition was also observed with elacridar and with verapamil but only at low concentrations[139]. These findings give us a valuable data of how sesquiterpenes bind within the binding pocket of P-glycoprotein.

Altogether, sesquiterpenes became an interesting new class of natural-derived P-gp inhibitors. They specifically interact with P-glycoprotein, thus avoiding possible side effects with other transporters. Besides, P-gp did not confer cross-resistance to sesquiterpenes and neither they interact with other transporters such as MRP1, MRP2 and BCRP; thus, transporters that cause multidrug

resistance phenotype do not affect absorption and distribution properties of sesquiterpenes. Finally, the dihydro-β-agarofuran skeleton provides a useful scaffold for rational design of new sesquiterpenes. Despite sesquiterpenes present a common core structure with similar substituents, P-gp inhibition capabilities span from pK_i (M) values over 6, for the most potent inhibitors to almost inactive compounds with pK_i values below 4.

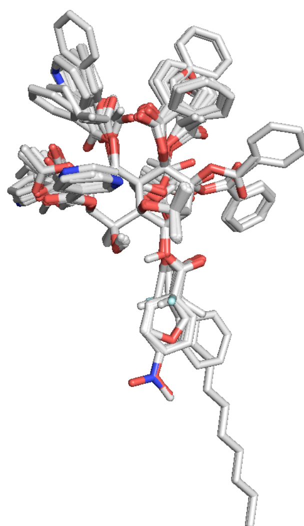

Figure 4. Superimposition of different sesquiterpenes by the dihydro-β-agarofuran skeleton.

3D- quantitative structure-activity relationships studies permit to gain insights into the structure requirements of sesquiterpenes as P-gp modulators. The different number and type of substituents along the rigid skeleton, give us the opportunity to study the key chemical features of P-gp–sesquiterpenes interactions by computational methods.

1.4 Comparative molecular similarity index analysis (CoMSIA)

3D-quantitative structure-activity relationships (3D-QSAR) methods are ligand-based approaches based in the assumption that affinity changes of compounds are based on their chemical features. By comparative molecular similarity index analysis (CoMSIA), the steric, electrostatic, hydrophobic, hydrogen bond acceptor, and hydrogen bond donor molecular fields of a set of compounds are calculated using a Gaussian-type distance dependence[141, 142]. It is assumed that the biological activity of compounds can be correlated and predicted by comparing these five molecular fields.

Molecular fields are determined by calculating the interactions of different probes at each point of a lattice intersection point of a spaced grid around the aligned ligands. At this point, a table of molecular interactions at different points of the space for each ligand is created; these are summarized to obtain the predicted activity values. The enormous amounts of molecular descriptors are reduced to a smaller set by partial least squares (PLS) method. PLS analysis attempts to identify the principal components that explain the maximum variance of the descriptors, and thus maximizing the correlation between biological and predicted activity values (pK_i, pIC_{50}, etc.). After, leave-one-out (LOO) and cross-validation methods evaluate the strength of the PLS model.

The values of the fitting coefficients can be represented as map contours of each molecular field around ligands, and displayed as different colors in order to facilitate visual inspection. The qualitative data of contour maps around ligands denotes key features of the ligands and summarizes protein-ligand interactions.

1.5 Molecular docking

Molecular docking is a computational method used to predict the preferred orientation of chemical compounds (generally small ligands) to a biological target, when they form a stable complex. Proteins, nucleic acids, enzymes etc. play a central role in signal transduction, which generally occurs after an external player, as a small molecule, interact with them. The interaction is usually highly specific, such that and only ligands with the correct chemical features and conformations can bind to biological targets. As a consequence the knowledge of the preferred orientation is of key importance to understand if and how a ligand will interact with a target. Docking algorithms works in two steps: conformational sampling and ranking. Once a set of feasible conformation is calculated (conformational sampling) docking algorithms also estimate the strength of the ligand-target complex, usually by means of theoretical algorithm called scoring functions, thus suggesting the most energetically viable conformations.

Ideally, conformational sampling should be extensive enough to reproduce experimental binding and the scoring functions should be accurate enough to rank the most viable conformations. The importance of the docking rely on the fact that the relative orientation of the ligands with respect to the target is of key importance in the activation/inhibition of the target and even the smallest changes in the chemistry or the conformation of the ligands may alter this equilibrium.

The great advantage of docking procedures is its relatively low investments and the rapidity the results are given. Plus, docking algorithm can virtually process millions of compounds and rank them; hence it has gained a fundamental role in rational drug design and discovery. Virtual screening of large dataset has became a key step in drug development, such that FDA included Bioinformatics and all its techniques in its Critical Path Initiative [143], with the aim to speed up drug discovery and to lower its development costs.

2. OBJECTIVES

Chapter 2.

OBJECTIVES

2.1 Biological Evaluation, Structure-Activity Relationships, and Three-Dimensional Quantitative Structure-Activity Relationship Studies of Dihydro-ß-agarofuran Sesquiterpenes as Modulators of P-Glycoprotein-Dependent Multidrug Resistance

It aims to understand the structural features of human P-glycoportein inhibition by sesquiterpenes compounds. The focus of this work is to perform a comparative molecular similarity indices analysis (CoMSIA) of a series of sesquiterpenes in order to identify the nature/position of most favorable substituents for human P-gp inhibition. These outcomes should guide the rational design of new sesquiterpenes compounds.

2.2 Sitamaquine Overcomes ABC-Mediated Resistance to Miltefosine and Antimony in *Leishmania*

To determine the binding modes of a series of drugs in a LMDR1 homology model and molecular docking studies. The MsbA and SAV1866 crystal structures are used as templates for homology modeling to study the feasibility of a semi-open inward facing state of P-gp.

2.3 Complete sequence analysis of ABCB transporters and computational analysis of P-glycoprotein inhibition by sesquiterpenes

This work is divided in two parts. First, a complete sequence analysis of ABCB family proteins, based on the structures alignment of available crystals, was performed. This led to the identification of some key conserved residues in each TMD. Second, the binding sites of a series of sesquiterpenes with a human P-gp homology model were investigated by analysis of molecular docking simulations.

3. PUBLICATIONS

Reyes CP, Muñoz-Martínez F, Torrecillas IR, Mendoza CR, Gamarro F, Bazzocchi IL, et al. Biological Evaluation, Structure−Activity Relationships, and Three-Dimensional Quantitative Structure−Activity Relationship Studies of Dihydro-β-agarofuran Sesquiterpenes as Modulators of P-Glycoprotein-Dependent Multidrug Resistance. J Med Chem. 2007 Oct 4;50(20):4808–17. DOI: 10.1021/jm070290v

Pérez-Victoria JM, Bavchvarov BI, Torrecillas IR, Martínez-García M, López-Martín C, Campillo M, et al. Sitamaquine overcomes ABC-mediated resistance to miltefosine and antimony in Leishmania. Antimicrob Agents Chemother. 2011 Aug;55(8):3838–44. DOI: 10.1128/AAC.00065-11

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Complete sequence analysis of ABCB transporters and computational analysis of P-glycoprotein inhibition by sesquiterpenes

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4. DISCUSSION

Chapter 4.

DISCUSSION

Computer-aided drug design has emerged as a valuable tool in medicinal chemistry. *In silico* methods provide an approach of protein-ligand interactions to explain the biological properties of chemical compounds and to guide the rational design of new drugs. P-glycoprotein is one the major goals of pharmaceutical industry due to its implications in multidrug resistance and ADMET properties of most drugs.

In this thesis, we studied the inhibition of P-glycoprotein by sesquiterpenes, a natural compounds extracted from *Celastraceae* plants. This class of compounds has been widely studied during the last years because of their structural variety and their multiple biological properties. They showed to be relative potent inhibitors of both human and *Leishmania* P-gp, reverting multidrug resistance phenotype in cancer cell lines and *Leishmania*. Interestingly, they showed no cross-resistance with other ABC transporters responsible of MDR in humans, such as MRP1 and BCRP; therefore, they became a promising new class of P-gp inhibitors.

After proving that sesquiterpenes interact within the TMD of P-gp, studies where designed to elucidate the chemical features that confer higher P-gp inhibition profiles. Dihydro-β-agarofuran sesquiterpenes are characterized by different esterifying substituents around a common skeleton. The relative position, number, and configuration of these residues, distinguishes each individual sesquiterpene.

In order to identify the structural requirements for P-gp inhibition by sesquiterpenes, we performed ligand-based studies of a series of sesquiterpenes proved to inhibit P-gp-mediated transport of daunomycin. We determined which substituents contribute the most to P-gp interaction by comparative molecular similarity index analysis (CoMSIA). Our 3D-QSAR model presented in this thesis showed the most outstanding key features for P-gp inhibition by sesquiterpenes, indicating the position of those substituents capable to make hydrogen bonds and hydrophobic interactions with P-gp.

The publication of *S.aureus* SAV1866 structure, opened the door to structure-based approaches. This structure, the first at high resolution, elucidated the correct TMD folding and secondary

structure of ABC exporters. SAV1866 structure was crystalized in its outward facing conformation, thus it represents a low affinity ligand binding state after release of solute. On the other hand, two bacterial MsbA structures were solved at inward facing conformations, in other words, in a conformational state of the transport cycle suitable for ligand binding. Later, the first crystal structure of a mammalian ABC transporter, the mouse P-gp, was published in an inward facing conformation. Mouse P-gp structure displayed a big volume between the two TMD with two cyclopeptides bound simultaneously.

The high identity level with human P-gp (87%) and the presence of ligands bound within the TMD, made of this structure the ideal template for human P-gp homology modeling. Nevertheless, it raised an open debate about whether the inward facing structures (showing wide NBD disassociation) were crystal artifacts or not, because some biochemical data indicated the impossibility of NBD to be so far apart under physiological conditions.

In this scenario, we built a homology model of *Leishmania* P-gp (LMDR1) based on the *S.aeurus* SAV1866 and *V.cholerae* MsbA structures. Two of the MsbA crystal structures were solved in inward facing conformations. While one of them was likely to be an artifact of crystallization conditions, due to the huge separation of NBDs displayed, the other crystal was in a semi-open state. The *V.cholerae* MsbA structure, despite opened to the cytoplasmic side, presented a much moderate NBDs disassociation. Unfortunately, the low resolution (5.5Å) of this crystal made impossible to use it as template for homology modeling.

Therefore, we built a homology model based on the MsbA structure but taking advantage of the secondary structure from the higher quality crystal of SAV1866 (3.0Å). Molecular docking studies of a series of drugs were performed using this homology model. Our results showed different binding pockets within the TMD of the semi-open model, where the rate of ligand interaction was different for each drug.

During the last years, the number of ABC exporters solved by crystallographic methods has been increased. The availability of new P-gp crystal structures and other bacterial transporters as well as the corrected model of the original mouse P-gp structure of 2009, revealed the large range of structural conformations that this family of proteins can adopt. Additionally, the recent crystal structure of mouse P-gp, with higher resolution than the previous (3.4Å), was

co-crystalized with four different ligands bound, showing that ligands can bind with distinct subsets of residues within the binding pocket.

We built a homology model of human P-gp using the new mouse P-gp structure as a template. Molecular docking simulations were carried out in order to determine the binding mode of a series of sesquiterpenes. Usually, analysis of ranked poses from molecular docking calculations requires further evaluation to discriminate the most viable binding poses. Instead of choosing of few docked results, we considered all the docked poses along each TMD of P-gp, and evaluated all protein-ligand contacts rates.

Analysis of the predicted protein-ligand interactions of a series of commercially available drugs was in agreement with bibliographic data. The methodology we used to determine the preferred binding site of each compound explained biological data, thus we used the same protocol to a set of sesquiterpenes of known biologically activity. Our results indicate that sesquiterpenes interacting deeper at the upper site of the TMD cavity (upper binding site) are the most potent inhibitors. The upper sites of P-gp are highly populated by hydrophobic and aromatic residues. Only two tyrosines and one serine are capable of making polar interactions with ligands.

Finally, the structural alignment of the ABC exporters available permitted us to create a multiple sequence alignment of a set of curated ABCB sequences. Penalty gap was increased in TMH regions of the structure profile alignment in order to preserve the conserved secondary structure of crystal structures. The aligned sequences, were analyzed and the most conserved residue of each TMH were identified. This information was used to propose a relative numbering scheme, which is a useful tool for comparison of distinct ABCB proteins.

5. CONCLUSIONS

Chapter 5.

CONCLUSIONS

1. The high level of polyespecificity showed by P-gp evidences different binding modes of ligands within P-gp, which can bind P-gp by different subsets of interactions. We have determined which substituents along the core skeleton of sesquiterpenes favor interactions with P-gp by a 3D-QSAR study, and hence, giving insights of the chemical modifications that could lead the design of new improved compounds.

2. The knowledge of the structure and mechanism of action of P-glycoprotein has increased during the last years. The availability of crystal structures opened the door to structure-based studies. We have performed molecular docking simulations of different drugs using a LMDR1 homology model based on a physiologically relevant conformation of MsbA transporter. Our results show the feasibility of using distinct homology modeling templates to study ligand interactions within the binding site of P-gp.

3. The structural data of ABC transporters has been greatly enriched by the increasing number crystal structures. Recent structural data confirmed the extent and plasticity of P-gp. We performed molecular docking simulations taking into account all possible interactions within the vast binding site of P-gp to study sesquiterpenes binding mode/s. Analysis of all protein-ligand interactions, by comparison with known drugs, showed to be a suitable method to evaluate docking results and to distinguish distinct ligand interactions.

4. The different crystal structures of ABC exporters showed a common topology. Their structural alignment allowed us to create a multiple sequence alignment of ABCB subfamily and to analyze their aminoacid conservation. Thus, we identified most conserved residues of each TMH and created a relative numbering scheme, which is a useful tool for comparison of distinct ABCB proteins.

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Chapter 6.

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7. APPENDIX

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