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Characterization of ABCB5 transporters in multidrug resistance mechanisms and their physiological functions

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Characterization of ABCB5 transporters in multidrug resistance mechanisms and their physiological functions

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Characterization of ABCB5 transporters in multidrug resistance mechanisms and their physiological functions

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Abstract

Background: ABCB5 is a member of the ATP-binding cassette (ABC) transporter superfamily. They mediate the ATP-driven transport a wide variety of substrates against their chemical gradient. Mostly, they have been studied for their implication in multidrug resistance (MDR). In human, they efflux out of the cancer cell or sequester in organelles different chemotherapeutics resulting in treatment failure. ABCB5 encodes several transcripts, the two longest ones encoding transporters, ABCB5FL and ABCB5β, respectively. Because of its conformation, ABCB5β needs to homo- or heterodimerize to become functional.

Aim: A recent study in our laboratory revealed two ABCB5 heterodimers with basal ATPase activity: ABCB5β/ABCB6 and ABCB5β/ABCB9. In the literature, there is some evidence that ABCB5 is involved in MDR, however, few studies have specified which isoform was investigated, rendering the interpretation of the data difficult. Therefore, we wanted to investigate the role of ABCB5 transporters and its interacting partners, ABCB6 and ABCB9, in MDR. Moreover, we decided to characterize the pharmacological profile of both transporter and further investigate their physiological function which, as proposed in the literature, could be linked with melanin production or transport.

Results: Due to transfection issues, we could only express GFP_ABCB5FL, GFP_ABCB5β and GFP_ABCB9 transporters in HEK293T and MelJuso, but no change in viability was observed when treated with increased concentration of doxorubicin. To avoid transient transfection, we then used a UACC257 cell line knockout for the ABCB5 gene in MTT assays to compare the effect of loss of expression of all ABCB5 isoforms on cell viability following treatment with doxorubicin, docetaxel, and paclitaxel. Surprisingly, UACC257 ABCB5 KO cells were more resistant to all three treatments than wild-type cells. Furthermore, we showed that the pharmacological profiles of ABCB5FL and ABCB5β are identical. Finally, we showed that ABCB5FL could be involved in melanin transport while no significative results were obtained regarding both isoforms implication in melanogenesis.

Conclusion: ABCB5FL and ABCB5β implication in MDR could not be determined, nevertheless, potential compensatory mechanisms were highlighted. On the other hand, both transporters showed similar pharmacological profiles. Finally, further investigations are needed regarding their implication in melanin transport and melanogenesis.

Keywords: ABCB5FL, ABCB5β, multidrug resistance, pharmacokinetic, melanin

Mémoire de master en sciences biomédicales

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Abbreviation list

ABC : ATP Binding Cassette
ABCB5FL : ABCB5 Full Length
AML : Acute Myeloid Leukemia
ATP : Adenosine Triphosphate
BBB : Blood Brain Barrier
BeFx : Beryllium Fluoride
BeSO4 : Beryllium Sulfate
CFTR : Cystic Fibrosis Transmembrane conductance Regulator
CNN : Convolutional Neural Network
DMSO : Dimethyl sulfoxide
DNA : Deoxyribonucleic Acid
FDA : Food and Drug Administration
5-FU : 5-Fluorouracil
GFP : Green Fluorescent Protein
HDL : High Density Lipoprotein
HEK : Human Embryonic Kidney
HIT : Haloperidol Induced Toxicity
Km : Michaelis Menten constant
KO : Knockout
LPI : Lysophosphatidilinositol
MDR : Multidrug Resistance
MITF : Microphthalmia-associated Transcription Factor
mRNA : Messenger Ribonucleic Acid
MTT : 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaF : Sodium Fluoride
NBD : Nucleotide Binding Domain
Pi : Inorganic Phosphate
P-gp : P-glycoprotein
PXE : Pseudoxanthoma Elasticum
S1P : Sphingosin-1-Phosphate
SNP : Synonymous Nucleotide Polymorphism
TAPL : Transporter associated with Antigen Processing-Like
TMD : Transmembrane Domain
TRP1 : Tyrosinase Related Protein 1
TYR : Tyrosinase
Vi : Vanadate
VLCFA : Very Long Chain of Fatty Acids
Vmax : Maximum Speed
X-ALD : X-linked Adrenoleukodystrophy

1. Introduction

1.1 ABC transporters

1.1.1 Generality

ATP-Binding-Cassette (ABC) transporters are among the largest protein superfamily expressed in all phyla of life [1,2]. These transporters are highly conserved through evolution [2]. In human, they are responsible for the unidirectional transport of a wide range of substrates, going from ions, peptides, vitamins, sugar, to larger molecules like polysaccharides or xenobiotics and play a variety of physiological functions [3–5]. Because they use the energy released by ATP hydrolysis to transport substrates against their chemical gradient, they are considered as primary active transporters [4].

ABC transporter superfamily is composed of seven different families named from A to G [6]. The 48 different ABC transporters were classified based on sequence homology, domain order and structure similarities [7]. In eukaryotes they are exporters, with some exception (i.e. ABCA4, ABCD4, ABCC7, ABCC8 and ABCC9), whereas in prokaryotes they can be found as exporters or importers, yet they are predominantly found as importers [1,4,7,8]. They are associated with the regulation of intracellular concentration of certain substances, rather than with the expulsion of these substances from the cell. ABC importers mediate transport of essential nutriments into the cytosol of prokaryotes, while exporters pump substrates out of the cell, from the cytosol to the extracellular compartment [6,7,9]. However, ABC transporters can be located in organelles, sequestering molecules inside, and not directly transporting them to the extracellular compartment. For example, ABCA3, which is localized in lysosomes, can sequester chemotherapeutic substrates within this organelle [10,11].

1.1.2 Topology

The typical topology of an ABC transporter consists of two transmembrane domains (TMD), each of them composed of six α-helices, and two nucleotide binding domains (NBD) also called ATP-binding cassettes (**Figure 1A**) [12]. In human, a full transporter is therefore composed of four functional subunits encoded by a single polypeptide [13,14]. This described topology is not found in all ABC transporters. Some of them are called “half-transporter” because they are only composed of one TMD and one NBD, like ABCG transporters [14]. Their NBD is found either at the N- or the C- terminal region (**Figure 1B-C**) [15]. Half transporters must either homo- or heterodimerize to become functional [12]. For what concern the B family, the NBD is located in the C-terminus of their TMD (**Figure 1B**) [7].

Moreover, some ABC proteins are not considered as transporters [2]. The families E and F are only composed of members with two NDB, without TMD, rendering the transport of compounds impossible [2]. They play a role in the mechanism of DNA translation [7]. Non-canonical ABC transporters, which are ABCC8 and ABCC9 have been described to have a regulatory role for ATP-sensitive potassium channel (Kir6.x) [4,16]. ABCC7 also called the cystic fibrosis transmembrane conductance regulator (CFTR) is an ATP-gated chloride channel [4,5].

Some ABC transporters share an unconventional topology: they are composed of an additional TMD, called TMD0, composed of five α helices (**Figure 1D**) [7,17]. For example, some members of the C family (ABCC1, ABCC2, ABCC3, ABCC6, ABCC10) have this supplementary TMD0, localized in the N-terminus of the protein [7,18].

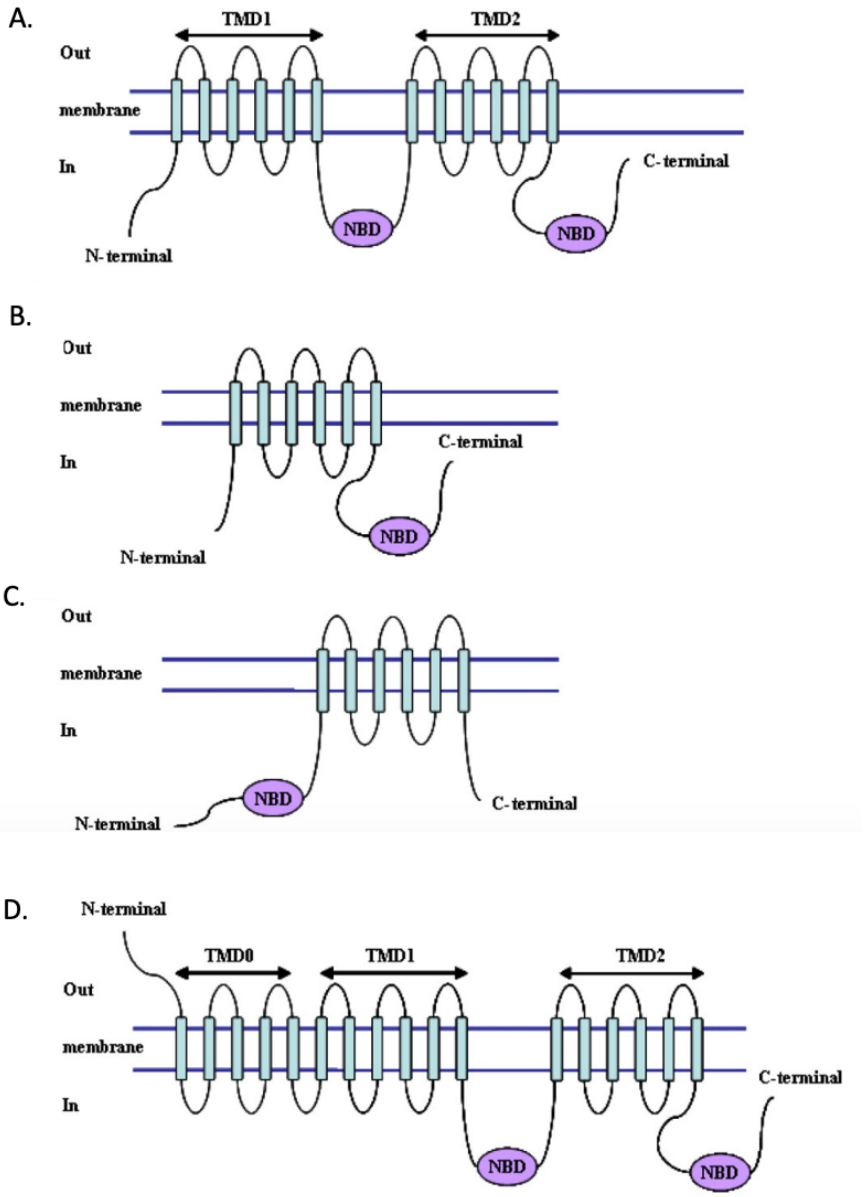


Figure 1 – Representation of ABC transporter 2D topologies. (A) 2D topology of a typical full transporter, with two transmembrane domains (TMD) and two nucleotide-binding domains (NBD). (B) 2D topology of a typical half-transporter, with one TMD and one NBD. (C) 2D topology of members of the family G. They have a reverse topology where their only NDB is localized in their N-terminus. (D) 2D topology of ABCC1, ABCC2, ABCC3 and ABCC6, members of the family C composed of an additional TMD, localized in their N-terminus. Modified from Gillet *et al* [15].

The TMDs are forming a pore into the lipid membrane, allowing the passage of substrates [4]. A substrate can be transported by an ABC transporter only if it is capable of interaction with the substrate binding pocket inside the TMD [19]. α-helices forming the TMD are responsible for the substrate specificity of the ABC transporter [6]. Although they are highly heterogeneous, TMDs share some characteristics within a transporter family [4]. Moreover, if a mutation occurs inside the binding pocket, changing one of its residues, the substrate specificity can be totally altered [16].

In contrast, the NBDs contain highly conserved motifs, separated into two subdomains: the catalytic core domain and the α-helical domain [7,9,20]. The central catalytic core domain contains motifs for ATP binding and hydrolysis, including the P-loop also called Walker A

motif, the Walker B motif, the Q-loop, the H-motif, and the D-loop (**Figure 2**) [17]. The α helical domain holds the signature for ABC transporters, a sequence of five amino acids (LSGGQ) [20]. The P-loop is involved in ATP binding and stability between ATP and substrate [17,21]. It contains highly conserved lysine residues. The Walker B motif facilitates energy transfer to the substrate. The Q-loop is a glutamate-rich sequence and plays a communication role between NBD domains. It coordinates ATP hydrolysis between the two NBD active sites. The H-motif is rich in histidine and is important for coordinating the metal ions required for ATP hydrolysis. Finally, the D-loop contains an aspartate and is involved in ATP binding and hydrolysis.

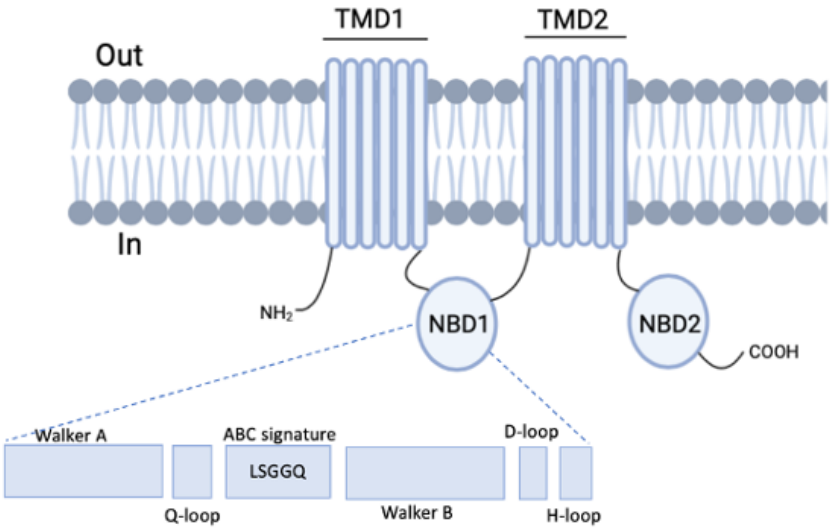


Figure 2 – Two-dimensional representation of the typical topology of ABC transporters. Structure of a typical full ABC transporter with two transmembrane domains (TMD) and two nucleotide-binding domains (NBD). The TMD are composed of 6 α helices crossing the membrane. The NBD are localized in the cytosol and are interlinked by a linker region between the first NBD and the second TMD. In the NBD are found highly conserved motifs, which are the Walker A motifs, the Q-loop, the ABC signature, the Walker B motif, the D-loop, and the H-loop, necessary for ATP binding and hydrolysis.

1.1.3 Transport mechanism – Catalytic cycle

ABC transporters are releasing substrates to the other side of cell membranes or sequester them in a organelle (depending on their localization) thanks to the energy released by ATP hydrolysis [21].

ABC transporter switches from outward-facing to inward-facing conformation through a catalytic cycle (**Figure 3**). However, the exact mechanism by which ABC transporters export substrates is not well known, even though some models have been described: (1) the alternating access model, which involved conformation changes between inward and outward states to transport substrates; (2) the ATP switch model, emphasizing ATP-induced conformation changes for substrate translocation; and (3) the constant contact model, where the substrate-binding site remains accessible on both sides of the membrane without major conformational shifts during transport [6]. The inward-facing conformation is the conformation where NBDs are widely separated in the cytosol, while when they dimerize the transporter adopts the outward-facing conformation [1,22].

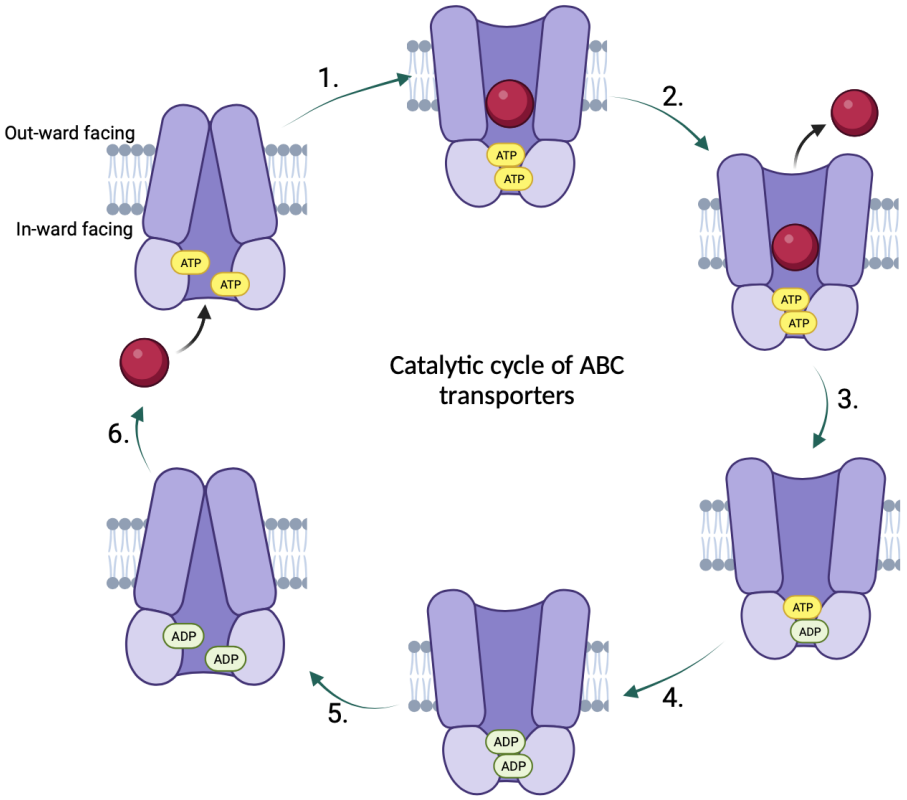


Figure 3 – Representation of the alternating model of the catalytic cycle of an ABC transporter where the export of a substrate takes place after the dimerization of NBD thanks to ATP binding and the following change in conformation after ATP hydrolysis. ABC transporter's catalytic cycle begins with the in-ward facing conformation where their two NBD are widely separated in the cytosol. The transporter will change conformation in the TMD, going to the out-ward facing conformation when a substrate binds into the binding pocket of the TMD. There two molecules of ATP will come to settle in each NDB, allowing their dimerization. Thanks to the energy contained in ATP, the substrate can be translocated, following ATP hydrolysis. The transporter will change conformation, allowing its reset.

The TMD/NBD interface is important to coordinate substrates translocation by transferring energy from the NBD to the TMD [13]. The catalytic cycle begins by the binding of the substrate into the binding pocket of the TMD (**Figure 3**) [4]. A molecule of ATP will bind to the catalytic core of each NBD, enabling a dimerization of the two functional subunits of the transporter [1,4]. At this stage, there is the switch in the TMD from the inward-facing conformation to the outward-facing conformation, hydrolyzing ATP and releasing a molecule of ADP and inorganic phosphate [4]. The energy collected from the hydrolysis of ATP will allow the substrate translocation, and the reset of the transporter by the dissociation of the NBD [1]. The transporter will return to its initial conformation, which is the inward facing conformation, to allow the transport of other substrates [1,4,22]. Actually, there are two substrates-binding sites in the TMD, which are switching from outward- or inward facing conformation, enabling the ABC transporter to functionate at all times [13,22].

1.2 ABCB family

1.2.1 ABCB5

ABCB5 is particular among the ABCB family because it exists under several isoforms [23]. The ABCB5 gene, localized on chromosome 7p21, gives rise to eleven transcript variants (**Figure 4**) [23]. Two encodes transporters, while the others encode soluble proteins. There is a full transporter (ABCB5FL and composed of 1257 amino acids), a half transporter (ABCB5β composed of 812 amino acids) and other small transcripts encoding for soluble proteins including ABCB5α, which is composed of 131 amino acids [23,24].

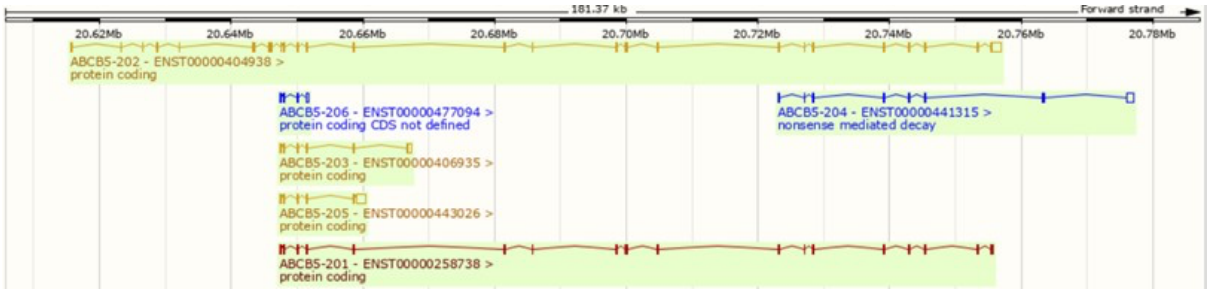


Figure 4 – Localization of the eleven transcripts of ABCB5 on chromosome 7p21. ABCB5-202 is ABCB5FL transcript of 1257 amino acids of a length of 5350 bp. ABCB5β is represented by ABCB5-201 transcript of 812 amino acids, and ABCB5α of 131 amino acids is shown on ABCB5-205.

ABCB5FL has the typical topology of a full transporter and is composed of two TMDs and two NBDs (**Figure 5**) [23]. On the other hand, ABCB5β is a half transporter but with an unconventional topology. It has one TMD and two NBDs, the N-terminus one lacking the conserved Walker A motif, necessary for ATP binding [23]. ABCB5β must either homo- or heterodimerize to become functional.

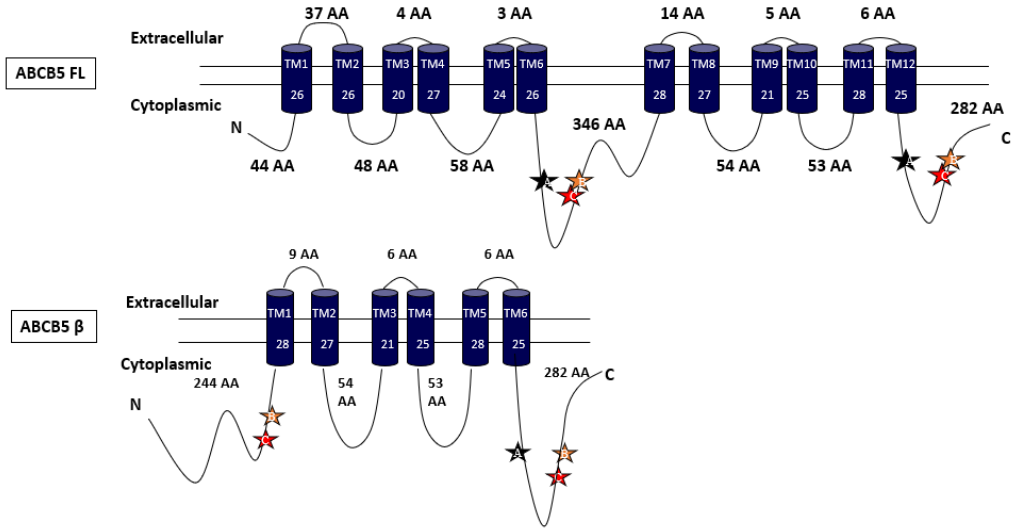


Figure 5 – 2D topology of ABCB5 two longest isoforms. ABCB5FL has the typical topology of a full transporter with two TMDs and two NDBs, where the stars represent patterns conserved in the NBD. ABCB5β has the unconventional topology to have one TMD and two NBDs. Its N-terminus NBD lacks the Walker A motif, described to be involved in ATP binding.

ABCB5 isoforms subcellular localization is still under investigation. ABCB5β was cloned from melanocytes, while ABCB5FL was cloned from cDNA libraries prepared from prostate and testis [23,25]. ABCB5β was shown to be a marker of skin progenitor cells, melanoma stem

cells and limbal stem cells [26–28]. Because ABCB5β is predominantly expressed in pigmented cells, it has been hypothesized that its physiological role must be linked to melanogenesis, the process which enable the production of melanin inside melanosomes [29]. Very recently, our laboratory unraveled the localization of ABCB5β in endoplasmic reticulum using HeLa and MelJuso cells [30].

ABCB5 has also been linked with multidrug resistance (MDR) in multiple cancer types, including malignant melanoma, breast cancer, colorectal cancer, hepatocellular carcinoma, head and neck, and leukemia [23,24,31]. In 2012, Wilson *et al.* highlighted the importance of ABCB5 in colorectal cancer [31]. In their studies they established a correlation between ABCB5 and resistance to 5-FU, which is the first line treatment for patients with colorectal cancer [31]. However, the study does not mention which isoform of ABCB5 is implicated in 5-FU resistance in colorectal cancer. Another study also mentions the fact that ABCB5 controls cancer proliferation in colorectal cancer [32]. This team also failed to distinguish between ABCB5 isoforms. Unfortunately, it is the case in many studies; only few of them specified which isoform of ABCB5 is investigated in multidrug resistance. In consequence it renders the interpretation of published data complicated and publication addressing separately each longest ABCB5's isoforms (i.e. ABCB5FL and ABCB5β) are needed.

Leung *et al*. have studied the genetic variations of ABCB5 and the associated risk in hepatocellular carcinoma [33]. They have identified three synonymous nucleotide polymorphisms (SNP) in ABCB5 that are associated with aggressive hepatocellular carcinoma characteristics, which are rs17143187, rs17143212 and rs2074000 [33]. These SNPs could be involved in the alteration of ABCB5 expression and mRNA stability [33]. An overexpression of ABCB5 gene is therefore linked with a poor prognosis in hepatocellular carcinoma but also in other cancer types. However, the underlying mechanisms leading to an increase in ABCB5's expression are not already known [33]. Recently, Sana *et al.* showed that ABCB5FL may have a role as a tumor suppressor in melanoma [34]. Its gene was found to be highly mutated in melanoma samples and these mutations led to decreased ATPase activity. They discovered that when ABCB5FL loses its function, the cells have increased proliferative and migration capacities in melanoma [34]. By contrast, ABCB5β can promote oncogenic activity [35].

Sugimoto's group has shown that ABCB5FL overexpressed in HEK293 cells lead to doxorubicin, paclitaxel and docetaxel resistance [36]. ABCB5FL is therefore able to confer resistance against taxanes and anthracyclines [36]. Another study performed by Keniya *et al.* pointed out that ABCB5FL was also able to mediate resistance to rhodamine 123, daunorubicin and clorgyline (**Figure 6**) [37]. Although ABCB5β must form a dimer to become a functional transporter, they showed that ABCB5β homodimer cannot confer drug resistance in the yeast model *S. cerevisiae* (**Figure 6**) and in mammalian cells [36,37]. Since these studies focused on a limited number of drugs, we cannot exclude the possibility that this homodimer is involved in drug resistance in melanoma or that it heterodimerizes to do so. Taking this into account, Tangella *et al.* have tried to characterize the binding sites of ABCB5FL based on molecular docking simulations using potential ABCB5's substrates (taxanes and anthracyclines) [24]. They were able to discover three putative binding sites involved in the binding of chemotherapeutics [24].

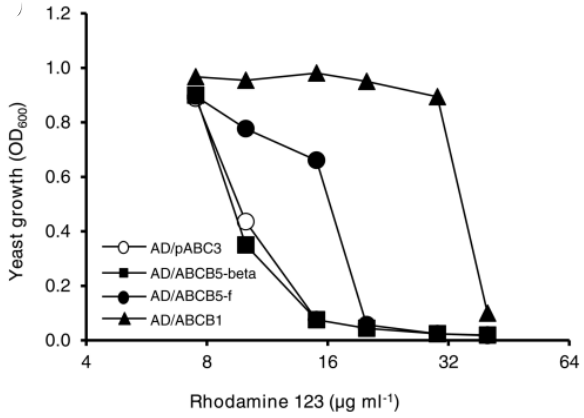


Figure 6 – ABCB5 resistance against rhodamine 123 in the yeast model *S. cerevisiae.* ABCB5FL confers resistance against rhodamine 123 in the yeast model *S. cerevisiae* like ABCB1, a close homologue of ABCB5, while ABCB5β do not mediate resistance, as shown in the negative control AD/pABC3. Rhodamine 123 exerts toxicity in yeast by affecting mitochondrial functions. Taken from Keniya *et al*. [37].

In our laboratory, Gerard *et al.* identified two interacting partners for ABCB5β by using three complementary techniques: NanoBRET, co-immunoprecipitation and proximity ligation assay [38]. Using two melanoma cell lines, MelJuso and UACC257, they discovered that ABCB5β can heterodimerize with ABCB6 and ABCB9, two other half transporters of the B family [38]. After having discovered these two heterodimers, they decided to investigate their ATPase activity. To do so, each interacting partner were fused together with a linker to force the heterodimerization in all possible orientation: ABCB5β_P-gp linker_ABCB6, ABCB6_P-gp linker_ABCB5β, ABCB5β_P-gp linker_ABCB9 and ABCB9_P-gp linker_ABCB5β. The P-gp linker is a 57 amino acid-peptide derived from ABCB1 (also known as P-gp) [38]. In Bathia *et al.,* they used the P-gp linker to study ABCG2 heterodimer, and they could demonstrate that the linker was not affecting the localization of the chimeric protein (ABCG2_P-gp_ABCG2) [39]. Gerard *et al.* were able to identify significant basal ATPase activity in the heterodimeric constructs mentioned above when expressed in high-five insect cells (**Figure 7**) [38]. So far, no substrate has been found for these transporters, however it is not excluded that ABCB5β heterodimerize with ABCB6 or ABCB9 to mediate resistance against chemotherapeutics.

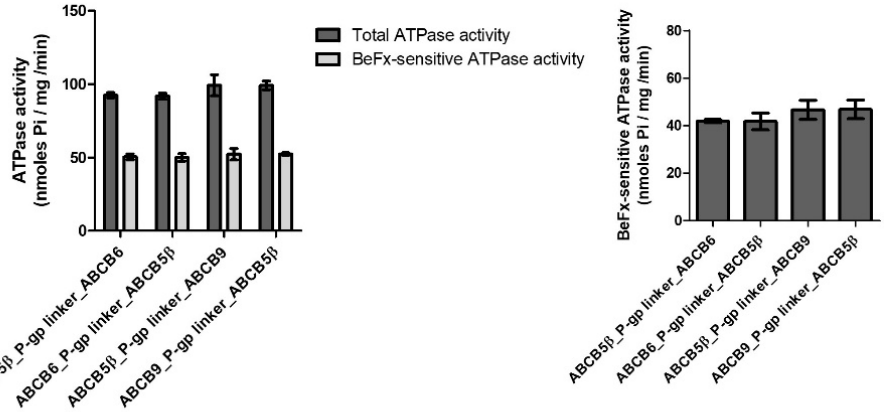


Figure 7 – Basal ATPase activity of ABCB5's heterodimers. On the left, ABCB5β_P-gp linker_ABCB6, ABCB6_P-gp linker_ABCB5β, ABCB5β_P-gp linker_ABCB9 and ABCB9_P-gp linker_ABCB5β showed significant basal ATPase activity in presence and in absence of BeFx. Beryllium fluoride (BeFx) can inhibit ATPase activity mediated by ABC transporters by interacting with the gamma phosphate of ATP. It forms stable complexes with ATP in their NBDs to prevent ATP hydrolysis. On the right is represented the BeFx-sensitive ATPase activity, when the ATPase activity detected in presence of BeFx is subtracted from the total ATPase activity. Taken from Gerard *et al.* [38].

1.2.2 Interacting partners of ABCB5

As reported by Gerard *et al.*, ABCB6 and ABCB9 (also named TAPL) are the interacting partners of ABCB5β [38]. Together they can form a heterodimer to become a functional protein.

ABCB6 and ABCB9 subcellular localization is under debate. They have been reported in the lysosomal membrane, the endoplasmic reticulum, the Golgi apparatus, the mitochondria and the plasma membrane [40,41]. These studies were performed in different cell types which might explain the heterogenous findings. For example, ABCB6 has been described to be localized in the mitochondrial membrane by quantitative mass spectrometry using subcellular fractions of ABCB6 [41,42]. Even though some studies have figured out a mitochondrial ABCB6 localization, other studies are claiming that ABCB6 is found in endolysosomal compartments [42,43].

ABCB6 was shown to regulate iron and therefore heme metabolism by transporting porphyrin metabolites at the inner mitochondrial membrane [40,41]. On the other hand, ABCB9 can transport oligo and polypeptides into the lysosomes and has already been described as being involved in decoupling activity [44]. This process happens when ATP hydrolysis is not correlated with TMD conformational changes, meaning transport of a substrate [44]. In the literature, ABCB9 has already been described to transport paclitaxel, leading to paclitaxel resistance [45].

Both ABCB6 and ABCB9 share an unconventional topology too. Beside one TMD and one NBD, they have a supplementary TMD in their N-terminus. For both transporters, this TMD, called TMD0, was shown to be involved in the localization of the transporter [38]. It has also been described that the TMD0 can function like an interaction hub for other proteins, to address the correct signalization of proteins into the lysosome [41,42]. The TMD0 of ABCB9 is composed of four transmembrane α-helices [41]. The TMD0 of ABCB6 is composed of five transmembrane α-helices at its N-terminus [41].

1.2.3 Other ABCB members

ABCB family is composed of eleven members, with the particularity to have both full and half transporters [5,7]. Three of them are full ABC transporters (ABCB1, ABCB4, ABCB11), while seven are half-transporters (ABCB2/TAP1, ABCB3/TAP2, ABCB6, ABCB7, ABCB8, ABCB9 and ABCB10) [40]. This family is characterized by having six α-helices in their TMD [7].

ABCB1, also called P-glycoprotein (Pgp) or multidrug resistance protein 1 (MDR1) is the most studied and characterized ABC transporter [15]. This transporter has a ubiquitous subcellular localization (in the plasma membrane, the endoplasmic reticulum, the Golgi apparatus, the endosomes, lysosomes and the mitochondria) [18,41]. It is expressed in many tissues and organs, including the liver, the placenta, the brain and the cortex, the kidney and in the colon [7,46]. Because of its ubiquitous expression, ABCB1 is responsible for many processes, such including in substrates' absorption and toxicity [18]. ABCB1 transports many substrates, including a wide range of chemotherapeutics which are exported out of cancer cells leading to drug resistance [5,46]. These drugs are mostly hydrophobic and belong to very different classes: such as anthracyclines, vinca alkaloids, taxanes, epipodophyllotoxins, and more [15].

ABCB2 and ABCB3 can heterodimerize in the membrane of the endoplasmic reticulum to become a functional protein [40]. They transport peptides into the endoplasmic reticulum which are then presented to the MHC-I dependent antigen presentation [5,40].

ABCB4 and ABCB11, two full transporters, are located in the liver, more specifically in the hepatocyte canalicular membrane [6,40]. They facilitate the secretion of bile acids into the biliary system, by transporting different components [5]. ABCB4 is responsible for phosphatidylcholine transport while ABCB11 transports bile acids [6].

ABCB7, ABCB8 and ABCB10 are known to be localized in the mitochondria and to be involved in heme biosynthesis by forming complexes with Fe/S protein to proceed with iron metabolism [5,40,43]. In cardiomyocytes, ABCB8 is part of the mitochondrial ATP-regulated potassium channel [5]. It is a subunit of this channel which can help reduce the damage caused by oxidative stress [17].

1.3 Physiological functions

ABC transporters are ubiquitously expressed rendering them very important for physiological functions. They play many roles for the correct function of organisms and mutation in one of these genes can cause severe conditions [3]. ABC transporters are implicated in membrane homeostasis, lipid trafficking (including bile secretion, β-oxidation and cholesterol transport), cell signaling, cell detoxification, drug resistance, immune response, therefore participating in intracellular regulation of organelles where they are expressed [3,5,6,46].

1.3.1 Substrates

Figure 8 shows a brief overview of physiological substrates for each ABC transporter's family. Some of them have a broad range of substrate specificity [3]. It is important to keep in mind that for several ABC transporters their physiological substrates remain unknown, for example ABCB5. ABCA family, composed of twelve members, is mainly responsible for the transport of lipids through membranes: phospholipids, cholesterol and bile acids [5]. The B family drives the export of a broad range of molecules, including iron, peptides, bile acids and drugs [5]. For what concern the C family, these transporters can translocate cyclic nucleotides, prostaglandins, glutathione, leukotrienes, bile acids, phospholipids, sulfates, and glucuronic acids [3,5]. Peroxisomal ABC transporters, belonging to the D family are necessary for the efflux of vitamin B12 and the transport of very long chain fatty acids through the peroxisomal membrane [5,14]. The G family is involved in the transport of lipids (phospholipids, bile acids, cholesterol), as well as cyclic nucleotides [5]. Only some members of the A, B, C and G families are responsible for multidrug resistance by exporting chemotherapeutics [3,5,15].

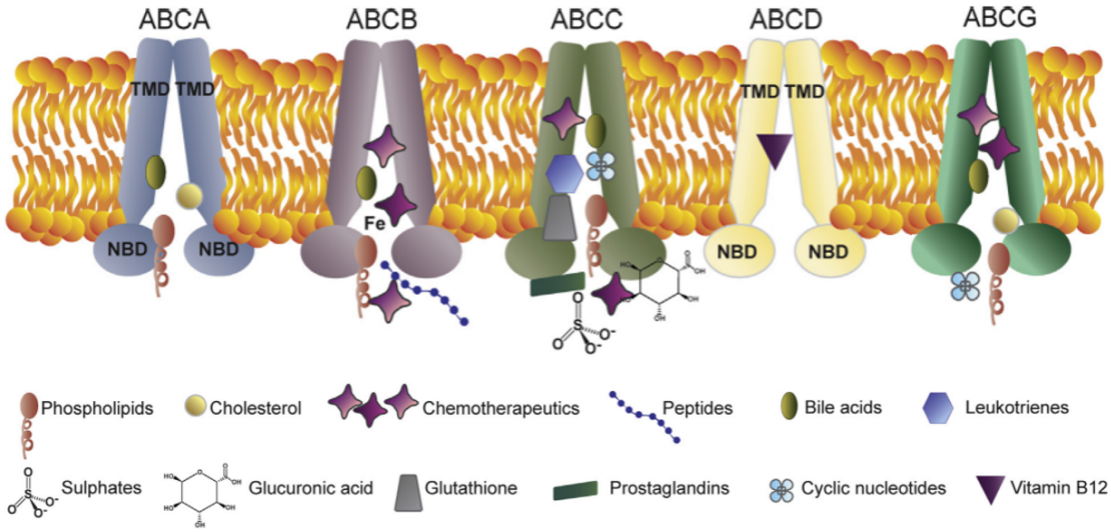


Figure 8 – List of substrate categories transported by the different families of ABC transporters. Endogenous molecules, drugs, and more, are transporters by families of ABC transporters depending on their TMD characteristics. Modified from Domenichini *et al* [5].

1.3.2 Pharmacokinetics

ABC transporters are involved in pharmacokinetics by playing a role in absorption, distribution, metabolism, and elimination of drugs (**Figure 9**) [3,18]. These parameters depend on the physicochemical properties of the molecules, therefore if a molecule is transported by any ABC transporter, its bioavailability will be affected depending on the ABC transporter's localization, and tissue distribution [3]. For example, ABCB1 is expressed in the enterocytes of the intestinal wall [18]. ABCB1 can pump drugs out of the luminal membrane, protecting enterocytes and intestinal cells, therefore limiting drug's absorption [7,18]. Hence, because of their localization in the luminal membrane of the intestine, liver and kidney, ABC transporters can regulate the absorption and distribution of molecules and drugs [18]. Another example is lysosomal ABC transporters. They can trap drugs into the lysosome, and limit the bioavailability of these molecules in the cell [41]. ABCB1 and ABCG2, localized in the brain epithelium, are part of the blood brain barrier, which is extremely important in protecting the brain from toxic substances, limiting their absorption (see section 1.3.3) [47].

ABCC3 is localized in the kidney, liver, and the gut, and mediates bile acids, glucoronate, and sulfate conjugates transport [18]. Therefore, its role is very important for the enterohepatic circulation, which allows an efficient reabsorption of substances (mainly bile acids) to enhance digestion and nutrient absorption in the digestive system [18]. This is an example of an ABC transporter involved in drug distribution.

Another example is ABCG2. It is expressed in the placenta, the blood brain barrier, the colon, the kidney, and more, rendering it important in absorption and distribution [48]. Because it is localized in the apical surface in the gastrointestinal tract, it supports the idea that it plays a role in the absorption of its substrates [48]. The inhibition of this transporter can interfere with drug distribution [48].

There is an indirect link between ABCB1 and cytochrome P450 CYP3A4, involved in the metabolism of most drugs [49,50]. ABC transporters fulfil interdependent roles with CYP450, affecting the rate at which a drug is metabolized [50,51]. Since ABCB1 has already been described to transport drugs, affecting their bioavailability, it can help to prolong drug exposure to metabolizing enzymes [50]. ABC transporters affect drug metabolism by influencing drug availability to hepatic metabolic enzymes in the liver, where most drug metabolism occurs [7], [50]. For example, ABCC2, located in the hepatobiliary tract, is involved in the metabolization and excretion of drugs conjugated with endogenous hydrophilic groups such as sulfates into the bile [5,40]. It has been shown that erythromycin, a macrolide agent, is a substrate of ABCC2 [51]. Based on that, Franke *et al*. have discovered that an impaired ABCC2 has direct consequence on the metabolization and elimination of erythromycin [51]. It therefore promotes the elimination of drugs metabolized by the liver [49].

Finally, ABC transporters are also involved in the elimination of drugs, xenobiotics, and endogenous compounds. ABCG1 can form stable complexes with cholesterol to promote its elimination [5]. Other ABC transporters are also located in the kidneys and bile ducts, influencing the excretion of molecules passing through the hepatobiliary tract [18]. This is also the case for ABCB1, present in the renal tubules, which can actively pump certain drugs and prevent their reabsorption, favoring their excretion in the urine [18].

ABC transporters are key components in drug development [52]. By designing drugs, scientists must be very careful about any interaction with ABC transporters [3]. It could have some severe effects on the drug's bioavailability and efficacy [52,53].

Figure 9 – Overview of pharmacological roles of ABC transporter in absorption, distribution, metabolization and excretion. This diagram demonstrates the importance of ABC transporters, preventing the absorption of toxins orally or by aerosol, and protecting highly regulated barriers such as the placenta or the brain. They are also involved in the distribution of molecules passing through the lung or the blood for example. ABC transporters are also involved in drug metabolism and elimination, being released in urine and feces, thanks to

their location in the kidneys and enterohepatic circulation. BBB, blood brain barrier; CSF, cerebrospinal fluid; BCFSB, blood cerebrospinal fluid barrier. Taken from Szakács *et al* [18].

1.3.3 Protection of sanctuaries

As introduced above, ABC transporters are very important to protect many sanctuaries in the body [18]. Some transporters are expressed in membranes where the permeability must be highly controlled. They play the role of protectors by exporting toxins, unwanted metabolites and cytotoxic drugs out of the blood brain barrier, the blood testis barrier, the blood placenta barrier and even the kidney, liver and gastrointestinal tract (**Figure 9**) [3,34,54]. It is one of their main functions as many ABC transporters are expressed in these vital tissues [18].

The placental barrier allows the passage of nutriments and oxygen, while preventing the absorption of toxins and rejecting waste products [47]. Because the slightest passage of a potentially toxic molecule through the placental barrier can be fatal for the fetus, the presence of ABC transporters (i.e ABCB1, ABCG2, ABCA1, ABCC1,…) for exporting these molecules is extremely important [47]. Their inhibition can lead to severe consequences for both the mother and the newborn.

The blood brain barrier (BBB) drastically regulates exchanges between the blood and the gray matter, allowing the passage of ions and nutrients necessary for the proper functioning of neurons, while preventing the passage of toxins and rejecting waste [47]. These exchanges are tightly controlled thanks to the presence of junctions made of occludins, claudins and zonula occludens [47]. Hydrophobic compounds can pass freely through membranes by passive diffusion but because of ABC transporters expressed in the blood brain barrier, they will be exported back to blood circulation [18,47]. In this barrier are expressed ABCB1, ABCC1, ABCC2, ABCC4, ABCC5 and ABCG2 with a predominance of ABCB1 and ABCG2 [18,47]. Other ABC transporters are also localized in the BBB, like ABCA2 and ABCA8, two proteins involved in lipid transport [47]. ABCB5 was also found to be in the blood brain barrier in a pharmacogenomic study addressing the susceptibility to haloperidol-induced-toxicity (HIT) [55]. Although loss of function mutations in the ABCB5 gene influence haloperidol concentration in the brain, the study did not reveal which of the ABCB5 isoforms is involved in this toxicity.

The presence of ABC transporters in the BBB could become a challenge when developing drugs to treat Alzheimer, epilepsy, or any disorder of the brain. ABC transporters will pump out these drugs to protect the brain, rendering it inactive at its site of action [18].

1.3.4 Lipid homeostasis

Both ABCA and G transporters are responsible for lipid transport and regulation of lipid homeostasis [5,19]. For example, ABCA1 regulate lipid homeostasis, communication, and movement [5]. It can translocate cholesterol to some precursors of HDL, by combining them with apolipoprotein A-I [56]. ABCG1 acts in the same way but latter in the process of lipid homeostasis by assembling cholesterol to already existing HDL particles [5]. Therefore, they are facilitating the elimination of cholesterol and they can both translocate phospholipids to the plasma membrane [5].

Peroxisomal transporters belonging to the D family (ABCD1, ABCD2, ABCD3 and ABCD4) can mediate lipid degradation into the peroxisome [14]. This degradation generates bioactive lipids that will be used for other processes [14]. In other words, they are involved in the

catabolic process of very long chains of fatty acids (VLCFA) using the β-oxidation to degrade them in carbon atoms [14,57]. ABCD1 mostly transports monosaturated fatty acyl-CoA, while ABCD2 has a broader substrate range including VLCFA and other lipid molecules like C20:0, C22:0, etc. ABCD3 is responsible for the degradation of branched chain fatty acids, bile acids precursors and dicarboxylic acids [57]. ABCD4 has been recently described in the lysosomal membrane to control the import of vitamin B12 rather than in peroxisomal membrane [14,17]. ABCD family is composed of only half transporters that must dimerize to become functional; they can be found as homo or heterotetramers [14]. Heterodimerization of ABCD1 with ABCD2 is functionally active, while an interaction of ABCD3 with these two latter's cannot function as an active transporter [14,57].

1.3.5 Disease condition

A mutation in ABC transporters that govern vital functions can generate disease conditions [3]. The mutation can impact the function of the transporter, its localization or even its substrate specificity if it affects the binding pocket of the TMD [6]. ABC transporters can be involved in many disease states affecting different tissues or to more complexed diseases like Alzheimer or gout disease [6]. In most cases, these mutations are linked with Mendelian inheritance [40]. The following are some examples of Mendelian diseases linked to ABC transporter malfunction (**Table 1**).

About 180 mutations in ABCA1 gene can be responsible for Tangier disease [3,56,58]. Among them, both missense mutation pArh937Val and pThr940Met can alter the first NBD of ABCA1, hindering the ability to bind ATP [58]. This disease is characterized by the absence of HDL in the circulation therefore leading to an accumulation of cholesteryl esters in the tissues [5,40,56]. A deregulation of ABCA4 gene involves the Stargardt disease, a rare condition affecting the vision [6]. ABCA4, expressed in photoreceptors, is responsible for the transport of retinol, commonly known as vitamin A [40].

A mutation in the ABCB4 or ABCB11 genes, two ABC transporters expressed in the liver, can lead to sever hepatic conditions [6]. Because they are transporting components of the bile, a recessive mutation of these genes can lead to hepatic bile acid accumulation, leading to hepatotoxicity and in more severe cases, fibrosis and cirrhosis [6].

ABCC6, expressed in the liver and in the kidney is responsible for a mineralization disorder called pseudoxanthoma elasticum (PXE), if a biallelic mutation alter its gene [6]. Several mutations in this gene can cause this condition and they appear most frequently in the second NBD of the transporter [59]. This condition affects conjunctive tissues and is characterized by the calcification of the elastic fiber of the skin, retina, and circulatory system [6]. A more known disease caused by a dysfunction in ABCC7 gene is the cystic fibrosis, the deadliest childhood ailment [40].

ABCD transporters are peroxisomal half-transporter mostly responsible for lipid trafficking [14]. Mutations in the ABCD1 gene is correlated with X-linked adrenoleukodystrophy (X-ALD), a disease reaching the white matter of the nervous system because of an accumulation of very long chain fatty acids into peroxisome of brain's cells [6,40]. A missense mutation in the substrate's binding pocket in the second TMD of ABCD1 frequently occurs, altering the lipid transport [60]. ABCD1 being on the X chromosome, women are carriers of the disease, while men are the most often affected [14].

Gene	Disease
ABCA1	Tangier disease, familial HDL deficiency
ABCA2	Intellectual developmental disorder
ABCA3	Pulmonary surfactant metabolism dysfunction type 3
ABCA4	Stargardt disease
ABCA12	Autosomal recessive congenital ichtyosis 4A
ABCB2, ABCB3	Immune deficiency
ABCB4, ABCB11	Progressive familial intrahepatic cholestasis
ABCB6	Dyschromatosis universalis hereditarian 3
ABCB7	X-linked sideroblastic anemia and ataxia
ABCC2	Dubin-Johnson syndrome
ABCC6	Pseudoxanthoma elasticum
ABCC7	Cystic fibrosis
ABCC8	Familial persistent hyperinsulinemic hypoglycemia of infancy
ABCC9	Intellectual disability with myopathy syndrome
ABCD1	X-linked adrenoleukodystrophy
ABCD3	Congenital bile acid synthesis defect 5
ABCG5, ABCG8	Sitosterolemia

Table 1 – Monogenic disease linked to human ABC transporters.

1.4 Cancer

1.4.1 Multidrug resistance

Among the 48 ABC transporters, the role of 13 of them in MDR has been fully characterized and it has been suggested that 20 other ABC transporters could also mediate multidrug resistance [54]. Drug resistance refers to the ability of cancer cells to resist to the effects of a treatment. It can manifest itself as a reduction in the sensitivity of a cell or organism to a drug, making the treatment less effective, whereas multidrug resistance (MDR) is defined as resistance of cancer cells to different anticancer agents with unrelated chemical structure and mechanisms of action [61,62]. There are several mechanisms responsible for resistance of a cancerous cells to treatment, naming enhanced drug efflux, increased DNA repair capacity, activation of detoxifying systems, evasion of apoptosis triggered by drugs, amplifications and epigenetic alterations [63,64]. These mechanisms are responsible for the reduction of the therapeutic efficacy of the drug administered, leading to treatment failure. This is why multidrug resistance is so problematic. For example, ABCB1 can participate to render a cancer cell resistant against chemotherapeutics, structurally and functionally different, by exporting them out of the cell or sequestering them into an organelle where it is expressed [63]. Moreover, as mentioned above, ABC transporters are involved in pharmacokinetics by participating in the metabolism of drugs. Because there is an indirect link between ABC transporters and CYP3A4, they can influence the rate of metabolism of xenobiotics [50,64]. Therefore, ABC transporters can influence the activation of detoxifying systems by increasing the frequency that a drug is metabolized by the CYP3A4. It will generate more metabolites that will be excreted, meaning that the drug could not act at its site of action, leading the cancer cell resistant.

Among all ABC transporters described to mediate multidrug resistance, ABCB1, ABCC1 and ABCG2 have been the most extensively studied [64]. ABCB1 has a wide subcellular

localization and mediates the transport of a broad range of molecules, including many chemotherapeutic agents [15,18]. ABCC1 is responsible for multidrug resistance by exporting different classes of drugs, from hydrophobic to amphipathic and non-ionic compounds [5]. ABCG2 is also fully described as a multidrug resistance protein but can also be involved in some physiological functions, for example as an urate exporter [5,48]. It can mediate the full resistance of methotrexate compounds, often used in clinic, as well as anthracyclines and epipodophyllotoxins [18].

In addition to severe mutations occurring in ABC transporter genes leading to genetic diseases, genetic variations called synonymous single nucleotide polymorphisms (SNPs) in these genes can influence ABC transporter's activity [54]. This may explain differences in the way individuals respond to anti-cancer drugs, contributing to variability in responses to chemotherapy. Therefore, these synonymous SNPs can lead to multidrug resistance mechanisms and difficulties to treat patients with anticancer agents. For example, more than 50 SNPs have been discovered for ABCB1 [64]. Gillet and Gottesman had highlighted the need to discriminate genes involved in multidrug resistance using sensitive and specific methods to better cope with these mechanisms [54].

Hypotheses have emerged that inhibiting an ABC transporter responsible for a disease could increase the sensitivity of a drug used to treat it [65]. However, this should be taken with caution because, as mentioned above, ABC transporters govern numerous physiological functions important for the organism, including oral availability, drug-drug interaction, and drug toxicity [3]. During the development process, the FDA now requires assessment of interactions between drugs and ABC transporters because ABC transporter inhibition can mediate undesired pharmacological outcome [3,19]. To modulate ABCB1, three generations of inhibitors have been described according to how their pharmacological effects change progressively over time [18,65]. The first generation include quinine and cyclosporine A [3,18]. Both have been showed to lack efficacy and then being toxic when increasing the dose to inhibit ABC transporter's function. To more specifically inhibit ABCB1, a second and a third generation of inhibitors have been developed, including valspodar and zosuquidar respectively [3,65]. Off-target inhibition due to interaction between inhibitor and cytochrome P450 3A4, the major phase I drug metabolizing enzyme, was the main problem of the second generation [3,18]. Therefore, zosuquidar, belonging to the third generation of ABCB1 inhibitors, has been designed to specifically target ABCB1 while not interacting with CYP3A4. As taken for example by Shaffer et *al.*, patients suffering from acute myeloid leukemia (AML) often overexpressed ABCB1 [65]. A clinical trial performed by ECOG3999 has demonstrated that inhibiting ABCB1 with third-generation inhibitor zosuquidar has no effect on patient survival from AML [66]. Actually, inhibiting ABCB1 to improve the outcome of patients with AML has often failed in clinical trials [65]. A number of questions may arise following the use of ABC transporter inhibitors [65]. How can we be sure that inhibiting ABCB1 will result in increased drug levels in leukemia cells ? Is there not a compensatory mechanism after inhibiting an ABC transporter, where other ABC transporters might overcome this inhibition and still allow the drug efflux ? The most important thing at the moment would be to understand all the mechanisms underlying ABC transporter inhibition as a therapeutic solution, rather than concentrating our efforts on getting inhibitors into clinical trials. In conclusion, other strategies has to be developed [18]. A promising strategy being nanoparticles loaded with anticancer agent used to bypass ABC transporters efflux and obtain a specific drug distribution [67].

1.4.2 Tumorigenesis

Most recently, ABC transporters were discovered to be involved in tumorigenesis. There are six hallmarks of cancer that have already been described by Hanahan and Weinberg [68]. Muriithi *et al.* have made a link between ABC transporters and the hallmarks of cancer (**Figure 10**) [19]. A dysregulated ABC transporter can drive cancer progression, indeed. For example, a poor expression of ABCA1, a transporter responsible for cholesterol homeostasis, is often linked with prostate cancer [19]. This down regulation is responsible for cancer proliferation because the cholesterol will not be transported out of the cells, resulting in an intracellular accumulation of lipids, conducive to cancer cell evasion [19]. On the other hand, cells overexpressing ABCB1 and ABCC1 have been described to be able to escape apoptosis, one of the hallmark of cancer [5]. Recently, Duvivier et *al*. have linked ABC transporters to the prospective new hallmarks of cancer and enabling characteristics proposed by Hanahan [69]. They have reported that ABC transporters can be responsible for these following processes, resulting in tumorigenesis: stimulating blood vessel formation, triggering invasion and metastasis, circumventing immune elimination, and eluding growth suppression, while maintaining proliferative signaling, resisting cell death, and disrupting cellular energetics.

In the same direction, because ABC transporters are responsible for lipid homeostasis by transporting lipids, prostaglandins, leukotrienes, cholesterol, cyclic nucleotides, sterols, sphingosine-1-phosphate (S1P), they are responsible for cancer progression [5,19]. By transporting signaling molecules important for cancer progression, they considerably promote tumorigenesis [19]. Lipids are a large source of energy available for many processes, therefore they can be used to generate molecules necessary for cancer cell growth or are used as signaling molecules to activate tumorigenic pathways [5]. These molecules are also used to promote or attenuate inflammation, a process that precede tumorigenesis [19]. For example, lysolipids, sphingosine-1-phosphate (S1P) and lysophosphatidilinositol (LPI) can be transported by ABCC1 [5]. These molecules are second messengers in signaling pathways underlying cancer development.

The composition of the plasma membrane is a good marker to discriminate against normal and cancer cells [5]. The lipid composition of cancer cells is distinct from healthy cells. Therefore, ABC transporters can maintain the correct composition of lipids into the plasma membrane of cancerous cells, therefore driving cancer progression [5]. ABC transporters can increase the level of unsaturated fatty acids which will be linked with a decrease in membrane fluidity [5].

ABC transporters are therefore known to be involved in the process of cancer proliferation by various mechanisms, although the underlying mechanisms are not yet fully understood [69]. In addition, there is an urgent need to identify the endogenous substrates of ABC transporters in order to understand the extent to which they may contribute to the development of cancer. A mutation in an ABC transporter can have a major impact on the way it binds its substrates. Much effort is currently being devoted to shedding light on this unanswered question.

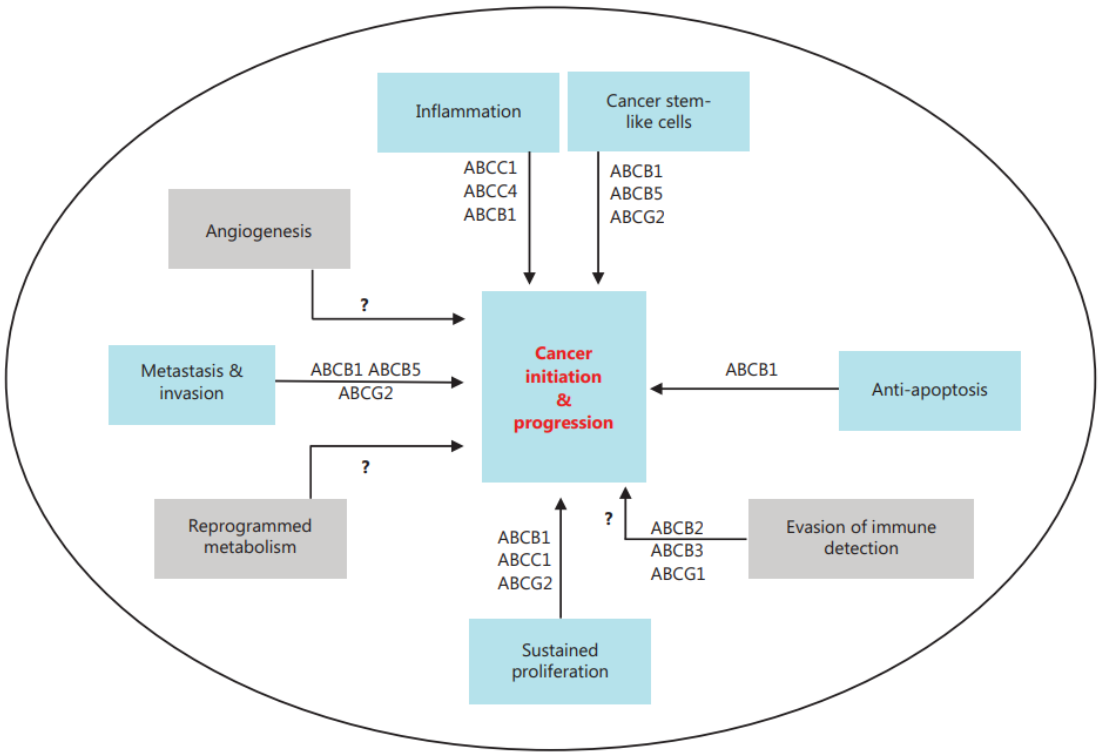


Figure 10 – Link between ABC transporter and cancer initiation and progression. So far, some ABC transporters have been described to play a role in tumorigenesis, by driving metastasis and invasion, sustained proliferation etc, features specific to cancer invasion. Taken from Muriithi *et al* [19].

1.5 ATPase assay

ATPase assay is the state-of-the-art assay to study the ATPase activity of ABC transporters. This assay is based on the quantification of inorganic phosphate released by the transporter after ATP hydrolysis (**Figure 11**) [53]. A potential substrate can modulate the ATPase activity of a transporter because uptake of substrates rely on ATP consumption [18,53]. However, it is important to note that ABC transporters possess intrinsic ATPase activity, which is not necessarily dependent on the presence of substrates [21]. As described before, if an ABC transporter mediates the translocation of any molecules, its NBDs will dimerize to engage the switch of conformation in its TMDs, following ATP hydrolysis [4]. For this reason, it is possible to characterize ABC transporters using ATPase assay.

To accurately characterize them, inhibitors are needed because of the intrinsic ATPase activity of ABC transporters [21]. This activity is measured by the difference in inorganic phosphate release in the presence and absence of an inhibitor, especially vanadate or beryllium fluoride [53]. These inhibitors are able to form stable complexes with ATP in NBDs, as they are phosphate analogues, and to prevent intrinsic ATP hydrolysis [70,71].

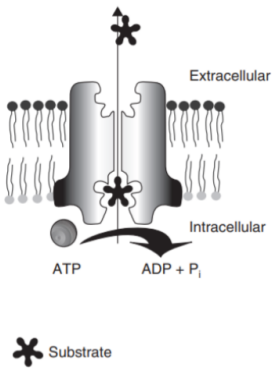


Figure 11 – Hydrolysis of ATP mediate substrate translocation. ATPase assays are based on the quantification of inorganic phosphate released after ATP hydrolysis. If a molecule happens to be a substrate of the ABC transporter, it will bind to the binding pocket inside of the TMD, allowing a dimerization of the NBD which is followed by ATP hydrolysis. Thanks to the energy released by ATP hydrolysis, the molecule can be transported across plasma and cellular membranes. This transport can be analyzed by the quantification of inorganic phosphate released. However, it is important to note that ABC transporters have a basal ATPase activity. Taken from Glavinas et *al.* [53].

The ABC transporter of interest is put in presence of ATP. If the molecule added a substrate of this transporter, the inorganic phosphate released by the ATP hydrolysis will form a complex that will turn blue following administration of Pi reagent and ascorbic acid (**Figure 12**) [72]. The absorbance measured is correlated with inorganic phosphate released but is not in direct correlation with the amount of substrate transported [53].

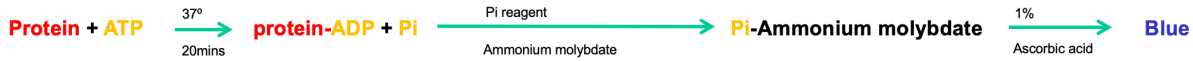


Figure 12 – Representation of the reaction behind the quantification of inorganic phosphate release in ATPase assay. The ABC transporters and the molecule tested as potential substrate are incubated 20 minutes at 37°C with ATP. ATP is hydrolyzed into ADP and inorganic phosphate (Pi) and Pi can interact with ammonium molybdate contained in the Pi reagent to form a molybdate-phosphate complex. Addition of 1% ascorbic acid will reduce the molybdate-phosphate complex to form blue complexes. The intrinsic ATPase activity of ABC transporter can also be tested without addition of specific substrate. It allows to see if the ABC transporter can hydrolyze ATP non-specifically. The absorbance will be read at 880 nm, synonymous of ATPase activity.

However, it is not because there is no detection of ATPase activity that the molecule tested cannot be a substrate of the transporter [46]. For example, it has been demonstrated that even though cyclosporin A is transported by ABCB1, ATPase assay does not reveal any increase in ATPase activity [53]. Therefore, the need of other experimental techniques to validate or invalidate the false positive or negative results, as proceeding cytotoxic assays in mammalian cells for example.

ATPase assays are a valuable tool in the pharmaceutical industry to detect drug interaction with ABC transporters, that could interfere with the bioavailability of a drug [50,53]. It can discriminate if a drug is a substrate or an inhibitor of an ABC transporter by measuring the amount of inorganic phosphate released [18]. Another use of ATPase assay is to characterize the transport kinetic of an ABC transporter by doing a Michaelis-Menten curve [46]. It can be done by varying the amount of substrate and ATP added with the transporter [46]. Therefore, this allows the identification of parameters intrinsic to an ABC transporter, such as its Km or Vmax. The Michaelis-Menten constant (Km) is the concentrations of ATP required to reach half of its maximum speed (Vmax), which quantifies the maximum transport capacity of the transporter.

2. Objectives

Three research axes were addressed in this master thesis to better characterize ABCB5 transporters. (1) Their implication in multidrug resistance mechanisms (MDR), (2) Their pharmacological characterization and (3) The investigation of their physiological functions.

2.1 Implication of ABCB5 transporters in MDR

As described before, ABCB5 is involved in MDR in melanoma. However, it is not clear whether ABCB5FL or ABCB5β (or both) is responsible of this resistance. To address the role of ABCB5β transporters, several constructs were prepared by Gerard *et al.*, knowing ABCB5β_P-gp linker_ABCB6, ABCB6_P-gp linker_ ABCB5β, ABCB5β_P-gp linker_ABCB9, ABCB9_P-gp linker_ ABCB5β [38]. These constructs were expressed in insect cells and the ATPase activity of these transporters was studied. Even though all the above-mentioned recombinant proteins showed basal ATPase activity, no change was seen following the addition of multiple chemotherapeutics (docetaxel paclitaxel, irinotecan, doxorubicin, 5-FU, vinblastine, and vincristine; data not shown). So far, three hypotheses can be raised. (1) none of these transporters (ABCB5β homodimer and heterodimers) transport the tested drugs; (2) the ATPase assay was not sensitive enough to show the change in ATPase activity; (3) ABCB5β transporters present a decoupled transport in which the substrate transport is independent of ATP hydrolysis.

To assess the three above-mentioned hypotheses, we wanted to study these transporters in mammalian cells. First, we had to generate the constructs of interest, see **Table 2**. To do this, we used the gateway cloning technology to insert cDNAs downstream a GFP tag in a pDEST53 plasmid. The gateway cloning includes the use of specific recombination sites called attB, attP, attR and attL which are recognized by specific enzymes known as integrase [73]. The BP clonase recombines attB and attP to produce attL and attR sites while LR clonase does the reverse reaction by recombining attL and attR sites (**Figure 13**).

1	ABCB5β_pDEST53
2	ABCB6_pDEST53
3	ABCB9_pDEST53
4	ABCB5β_Pgp linker-ABCB6_pDEST53
5	ABCB6_Pgp linker- ABCB5β_pDEST53
6	ABCB5β_Pgp linker-ABCB9_pDEST53
7	ABCB9_Pgp linker- ABCB5β_pDEST53
8	ABCB5FL_pDEST53

Table 2 - Constructions generated using the gateway cloning technology technique. Different constructions generated by gateway cloning for a better interpretation of the previous results obtain by Gerard *et al.* after ATPase assays.

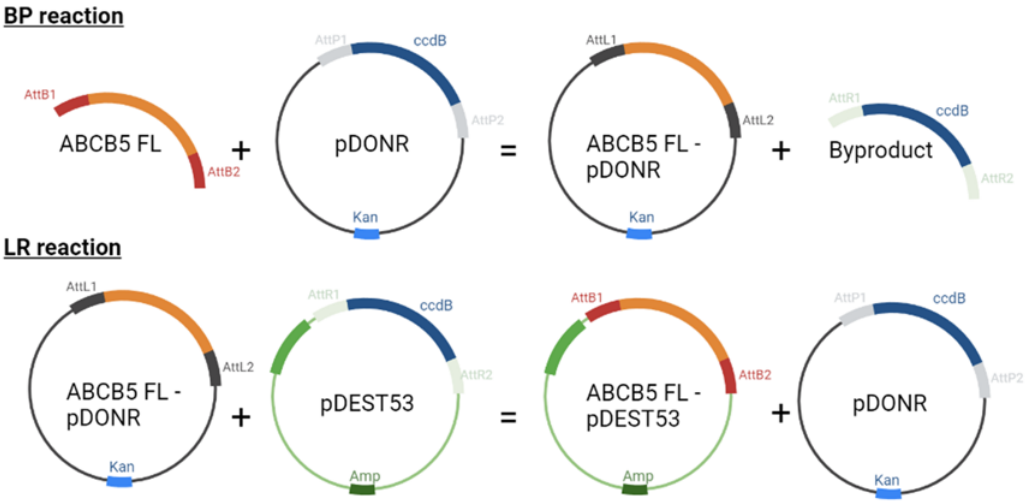


Figure 13 - Schematic representation of the gateway cloning technology. After amplification of the fragment of interest (ABCB5FL) by PCR to add att sites (represented in red), ABCB5FL sequence is transferred into a pDONR plasmid by the recombination of attB sites with attP sites (represented in grey) by the BP clonase. For the LR reaction, pDEST53 plasmid with a GFP tag is combined with the pDONR plasmid. Using a LR clonase, attL sites (represented in black) are recombined with attR sites (represented in green). At the end of the reaction, the plasmid of interest, resistant to ampicillin, has ABCB5FL cDNA cloned downstream to the GFP sequence. All plasmid containing the ccdh sequence will die as its expression is lethal in bacteria.

Then, MTT assays were performed on mammalian cells overexpressing the recombinant proteins to unravel potential resistance mediated by these transporters after addition of chemotherapeutics. The MTT assay evaluates the viability of cells using yellow tetrazolium salt [74]. If the cells are still alive, their active enzymes localized in mitochondria will reduce the tetrazolium salt into purple formazan salt. After solubilization using DMSO, purple crystals can be quantified by reading the absorbance at 570nm. The more purple the medium is, the more intense is the absorbance and healthier the cells are. As described in the introduction section, if the drugs are transported by the transfected ABC transporter, it will either be exported out of the cells or sequestered in an organelle leading to survival of cancer cells which will be determined by an increase of the IC50 value of the tested drug when compared to mock-transfected cells. These results could validate or invalidate the three hypotheses we formulated.

2.2 Pharmacokinetic characterization

Since there is little information on ABCB5FL and ABCB5β transport function in the literature, we decided to better characterize them by using ATPase assay, the state-of-the-art approach to study the ATPase activity of ABC transporters. In this project, the ATPase activity of ABCB5 transporters was studied when expressed in insect cell membrane vesicles.

To do that, ABC transporters of interest were expressed in insect cells (**Figure 14**). Using an ultracentrifugation protocol, we harvested insect cell total membranes expressing the transporter of interest. To express them in insect cells, each cDNA of interest was cloned into a bacmid. Then, Sf9 insect cells were transfected with the bacmid of choice. The transfected cells produced baculoviruses, which were used to infect high-five insect cells. It led to the expression of the transporter of interest. Sf9 cells were only used for baculovirus production because they can easily be transfected, while their protein expression is relatively modest [75]. On the other hand, high-five cells are much harder to transfect but lead to high recombinant protein expression [75].

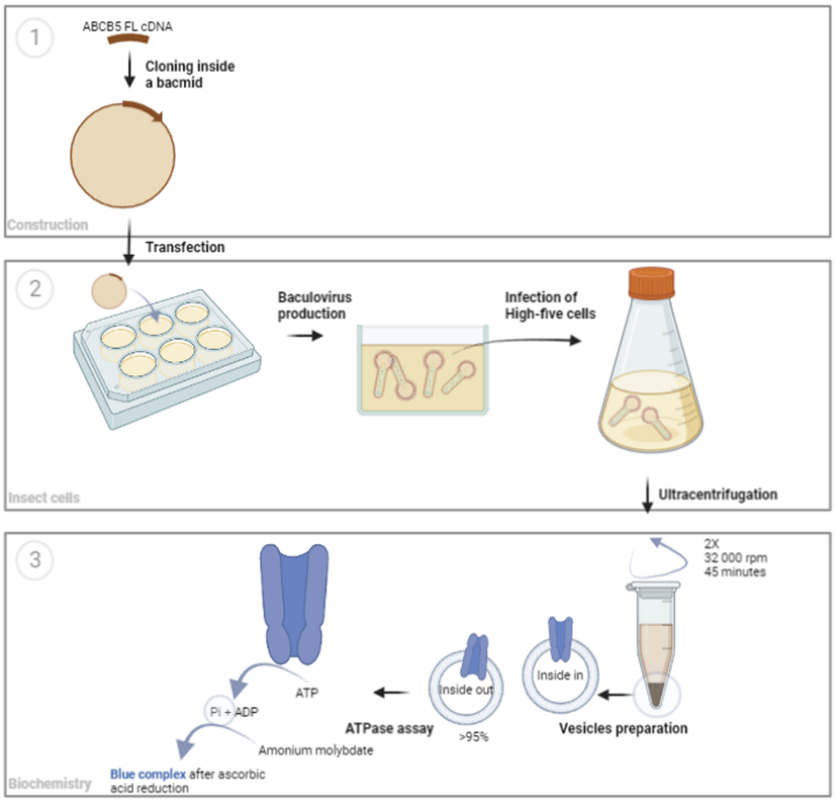


Figure 14 - Schematic layout of ABCB5FL expression in insect cells and vesicles preparation. To express ABCB5FL in insect cells, its coding sequence must be introduced into bacmids, which are then transfected into Sf9 insect cells. Following transfection, Sf9 produce baculoviruses. These baculoviruses are harvested and used to infect High-Five insect cells. After the expression of ABCB5FL in High-five cells due to baculovirus infection, cells are lysed, and membranes are prepared using ultracentrifugation. Two types of membrane vesicles are prepared: inside-in and inside-out. The inside-out population represents more than 95% of the membranes obtained. Then, the collected vesicles are used to perform ATPase assays. ATP hydrolysis can be quantified by the amount of inorganic phosphate (Pi) released after ATP consumption. Blue complexes are created in the presence of ammonium molybdate and ascorbic acid.

As mentioned earlier, the literature lacks studies looking at ABCB5β and ABCB5FL separately. To compensate, we decided to perform a complete pharmacokinetic analysis of both transporters separately using the total membrane vesicles from high-five insect cells expressing the transporter of interest. This pharmacokinetic characterization includes inhibitor curve, Michaelis-Menten curve and thermostability of both transporters. Overall, it will allow to better identify if these two isoforms of ABCB5 have differences in their transport function.

2.3 Investigation of ABCB5 physiological function

ABCB5 transporters are both expressed in pigmented cells such as melanocytes, which led to the hypothesis that they might be involved in melanin production. To investigate this, UACC257 KO for ABCB5 longest isoforms, ABCB5FL and ABCB5β using CRISPR technology were obtained from Cyagen (Santa Clara, USA). Exon 13 of ABCB5 gene has been selected as target site. After verification of the correct suppression of ABCB5 expression, melanin content of UACC257 KO cells was compared to the wild type couterpart. Next, melanin content of UACC257 overexpressing either ABCB5β or ABCB5FL was analyzed. Then, the expression of different proteins involved in melanin production (TRP1, TYR and MITF) was evaluated. All together, these experiments helped us determine whether ABCB5 could be involved in melanin production, and which isoform is involved in this mechanism.

3. Materials and Methods

3.1 Gateway cloning

3.1.1 LR reaction

150ng of entry vector (pDONR201 constructions from Gerard *et al.* [38]), 150ng of destination vector pDEST53 or pDEST47 (ThermoFisher Scientific, Waltham, USA) and TE buffer were mixed to reach a final volume of 8µL. The LR clonase enzyme (ThermoFisher Scientific, Waltham, USA) was thawed on ice for 2 minutes and vortexed twice for two seconds. Then, 2µL of enzyme was added to the reaction and the mix was incubated overnight (approximatively 18 hours) at 25°C. Next, 1µL of proteinase K was added to the mix and the samples were incubated at 37°C for 10 minutes to stop the reaction. 2µL of the reaction were transformed in 5-alpha competent *E.coli* following manufacturer's instructions (New England Biolabs, Ipswich, USA). The transformation was performed on agar plates with ampicillin.

3.1.2 Screening of colonies

An average of 23 colonies were screened after each transformation. A polymerase chain reaction was performed with the GoTaq polymerase (Promega, Madison, USA), and proper primers (**Table 3**) using the following cycles: 95°C 2 minutes, 25-time 95°C 30 seconds, 57°C 30 seconds, 72°C 2 minutes 30 seconds (or 4 minutes depending on the size of the sequence to be amplified) and 72°C for 5 minutes. The PCR products were loaded on a 1% agarose gel with ethidium bromide for about 20 minutes at 120V. The detection was performed using BioRad Gel Doc XR (Bio Rad, Hercules, USA).

Primers name	Primers sequence
GFP_Forward	ATGGCCAGCAAAGGAGAAGAAC
ABCB5β _Reverse	TCACTGCACTGACTGTGC
ABCB5β _Forward	ATGGTGGATGAGAATGACATC
ABCB6_Reverse	TCACCGTTCCATGGTCTG
ABCB6_Forward	ATGGTGACTGTGGGCAAC
ABCB9_Reverse	TCAGGCCTTGTGACTGCC
ABCB9_Forward	ATGCGGCTGTGGAAGG
ABCB5 FL_Reverse	TCACTGCACTGACTGTGCATT

Table 3 - List of primers (reverse and forward) used to screen colonies. Sequence of reverse and forward primers of GFP, ABCB5β, ABCB6, ABCB9 and ABCB5FL. GFP_Forward and the corresponding reverse primer were each time used to screen pDEST53 colonies to detect the correct ones. For the detection of pDEST47 colonies, ABCB6_Forward and ABCB6_Reverse primers were used to detect ABCB6_pDEST47, ABCB5β_Forward and ABCB6_Reverse primers were used to detect ABCB5β_P-gp_ABCB6_pDEST47, and ABCB9_Forward and ABCB5β_Reverse primers were used to detect ABCB9_P-gp_ ABCB5β_pDEST47.

3.1.3 Plasmid DNA miniprep

One correct colony per construct was added to 3mL of LB medium with ampicillin overnight at 37°C and 180 rpm. The amplified plasmid was harvested using a PureYield Plasmid Miniprep System kit following manufacturer's instructions (Promega, Madison, USA).

3.2 Cells culture and baculovirus titration

Immortalized human embryonic kidney cell line (HEK293T) and human melanoma cell lines (MelJuso and UACC257) were cultured using Dulbecco's Modified Eagle's Medium (DMEM)

(VWR, Pennsylvania, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Saint-Louis, USA). For insect cells, SF-900 III SFM (ThermoFisher Scientific, Waltham, USA) medium was used, supplemented with 10% FBS for Spodoptera frugiperda ovarian cells (Sf9) or without for Trichoplusia ni ovarian cells (high-five). Cells were maintained at 27°C on a shaker at 138rpm.

Baculovirus titer was determined using Sf9-Easy Titer (Sf9-ET) cell line. Baculovirus are diluted in a 96-well plate (1:1000, 1:5000, 1:25000, 1:125000, 1:625000, 1:3125000, 1:15625000, 1:78125000, 1:390630000) with $7{,}5.10^4$ cells per well and fluorescence is analyzed by microscopy. After baculovirus harvest, high-five insect cells were infected at a MOI (multiplicity of infection) of 10.

3.3 Transfection

Cells were platted in a 6-well plate to reach a confluence of 70% at the time of transfection. For insect cells, the FuGene transfection reagent protocol from Promega (Madison, WI, USA) was followed with 1µg of DNA in a 6-well plate. For HEK293T and MelJuso, FuGene from Promega (Madison, WI, USA) was used with 2µg of DNA, and the transfection was performed following the manufacturer's instructions. UACC257 were transfected with JetPrime (Polyplus, Illkirch, France) using 1µg of DNA following the manufacturer's instructions. Insect cells were harvested after 96 hours, while mammalian cells were harvested 72 hours post transfection.

3.4 MTT assays

For HEK293T cells, 2000 cells per well were platted in a 96-well plate and treated for 72 hours with doxorubicin diluted in DMSO (**Table 4**). For UACC257 cells, 5000 cells were added per well, treated 72 hours with doxorubicin, or 24 hours with docetaxel and paclitaxel (**Table 4**). After time of treatment, 20µL of 5mg/mL MTT was added to the plate and incubated at 37°C for 3 hours. Then, medium was removed and 150µL of DMSO were added to the cells. Plates were incubated on an orbital shaker for 15 minutes. The absorbance was read using a Spectramax i3x (Molecular Devices, San JoSe, USA) at 570 nm.

Concentration of doxorubicin used for HEK293T cells (nM)	Concentration of doxorubicin used for UACC257 cells (nM)	Concentration of docetaxel used for UACC257 cells (µM)	Concentration of paclitaxel used for UACC257 cells (µM)
2 500	10 000	600	150
833	5 000	300	100
277	2 500	200	75
93	500	100	50
31	250	75	25
10	50	50	12.5
3.4	5	37.5	6.25
1.14	2.5	25	3.13
0.4	1.75	20	1.04
0.13		10	0.5
0.04		5	
0.014		2.5	
		1.25	

Table 4 – Concentrations of doxorubicin, docetaxel and paclitaxel used to treat HEK293T and UACC257 cells.

3.5 Proliferation assay

2000 cells per well were seeded in a 96 well plate. At four different time point (0, 24, 48 and 72 hours), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT solution) was added to a final concentration of 0.5 mg/ml and cells were incubated for 3 hours at 37°C. next, medium was removed, and cells were solubilized in 100µl of DMSO. After 15 minutes incubation, absorbance was read at 570 nm with a SpectraMax i3 (Molecular devices, San Jose, USA).

3.6 Western blot

Samples were diluted in 5X blue loading dye, centrifuged, vortexed, and heated for 10 minutes at 40°C. The amount of proteins was determined after protein dosage using the Pierce™ BCA Protein Assay Kit from ThermoFisher Scientific (Waltham, MA, USA). After loading on a 8% polyacrylamide gel, samples were migrated at 120V. The transfer on a PVDF membrane was performed at 110V for 1h30. The membrane was incubated for 30 minutes in 5% milk (diluted in PBS 0,05% Tween 20), then overnight at 4°C in the corresponding primary antibody (anti-ABCB5 Rockland, anti-ABCB1 (C219) Fujireblo, anti-TRP1 Abcam, anti-MITF Abcam, anti-tyrosinase Santa Cruz). After 3 washes in PBS 0,05% Tween 20, the PVDF membrane was placed in 1/5000 Scan Latter anti-mouse or anti-rabbit secondary antibody, diluted in 5% milk in PBS 0,05% Tween 20, for one hour (Molecular Devices, San Jose, USA). Three washes in PBS 0,05% Tween 20 were performed again, and the membrane was read using Spectramax i3X (Molecular Devices, San José, CA, USA).

3.7 Insect cell total membrane preparation

50 to 72 hours post baculovirus infection high-five insect cells were centrifuged at 3000 g for 15 minutes at 4°C and the pellet frozen at -80°C. Every step was carried out at 4°C. Pellets were resuspended in the homogenization buffer (50mM Tris HCl pH 7.5, 50mM D-mannitol, 2mM EGTA, 2mM DTT, 1mM AEBSF and 0,5% aprotinin) and kept on ice for 30 minutes. Cells were then passed 40 times in a loose-fitting pestle, following 40 additional passes in a tightly fitting pestle. Then, cells were centrifuged at 2000 rpm for 10 minutes, and the supernatant was transferred in ultracentrifuge tubes. This step was carried out twice. Cells were spined at 38000 rpm for 53 minutes at 4°C using a 70 Ti rotor. Then, pellets were resuspended in resuspension buffer (50mM Tris HCl pH 7.5, 300mM D-mannitol, 1mM EGTA, 1mM DTT, 1mM AEBSF, 0,5% aprotinin) using bent needles, and centrifuged at 38000 rpm for 53 minutes at 4°C. The supernatant was discarded and membranes in the pellet were homogenized using storage buffer (50mM Tris HCl pH 7.5, 300mM D-mannitol, 1mM EGTA, 1mM DTT, 1mM AEBSF, 0,5% aprotinin, 10% glycerol) using bent needles. Membranes were stored at -80°C.

3.8 ATPase assays

Total membrane vesicles were prepared in an ATPase assay buffer (100mM MES-Tris pH6.8, 100mM KCl, 10mM sodium azide, 2mM EGTA, 2mM ouabin, 20mM $MgCl_2$ and 4mM DTT) to obtain a final concentration of 10µg of protein/0,1 mL. Membrane vesicles were placed in glass tubes with 3µL of inhibitor, BeFx (0,2mM beryllium sulfate and 2,5mM sodium fluoride) or 0,3mM vanadate and 1µL of DMSO or tested substrate were added. Glass tubes were incubated 2 minutes at 37°C. The reaction started with addition of 5mM ATP for 20 minutes at 37°C. After that time, the reaction was stopped with 100µL of 10% SDS. 400µL Pi reagent (1% ammonium molybdate in 2,5N sulfuric acid and 0,014% antimony potassium tartrate), 500µL of water and 200µL of 1% ascorbic acid were added. After 10 minutes of incubation, absorbance was read at 880nm using a Spectramax i3x (Molecular Devices, San José, USA).

3.9 Total melanin content dosage

Cells were washed twice in PBS and counted using trypan blue in TC20 automatic cell counter (Bio Rad, Hercules, USA). Lysis buffer (120 mM NaCl, 50 mM Tris, NP40 0.5%, 5 mM EDTA, 1x protease inhibitor) was added on the cells for 30 minutes then cells were centrifuged at 10000g for 10 minutes at 4°C. Protein dosage using the Pierce BCA Protein Assay Kit from ThermoFisher Scientific (Waltham, MA, USA) was performed on the supernatant and 200µL of NaOH 1M was added on the pellet. The pellet was heated at 60°C for 2 hours. Absorbance at 415nm was read using a Spectramax i3x (Molecular Devices, San José, USA). The total melanin content per mg of protein was normalized using a standard curve of synthetic melanin (Sigma-Aldrich, Saint-Louis, USA), ranging from 500µg to 0µg.

3.10 Molecular docking

Molecular docking analysis was performed by CD ComputaBio (New-York, USA). ABCB5 3-dimension model was constructed manually using as template the mouse model of ABCB1 (PDB:5KPI). Molecular docking analysis was performed using the Maestro software. Melanin structure was taken from pubchem.

3.11 Bacmids construction

ABCB5FL and ABCB5β coding sequences were fused following NEBuilder HIFI DNA Assembly manufacturer's instructions (New England Biolabs, Ipswich, MA, USA). The resulting sequences were cloned in pDONR201 plasmid using the SACII restriction enzyme, the sequence derived from P-gp flexible linker region residues (NEVELENAADESKSEIDALEMSSNDSRSSLIRKRSTRRSVRGSQAQDRKLSTKEALD) 703N-760D, was inserted between each of the half transporters. DNA sequencing was used to confirm both the correct orientation of the linker and the absence of other mutations. Next, sequences were transferred in pDEST-008 plasmid. Bacmids were generated from the pDEST-008 plasmid using Max Efficiency DH10Bac competent cells (ThermoFisher Scientific, Waltham, Massachusetts, USA). DH10Bac competent cells were transformed and spread on agar plates containing kanamycin, tetracyclin, gentamycin, Xgal, and IPTG for the selection of positive colonies. White colonies were selected for PCR with M13 primers (forward – cccagtcacgacgttgtaaaacg, reverse – agcggataacaatttcacacagg) in order to detect colonies that underwent correct transposition. M13 sites are localized on each side of the transposition site (around 300 bp).

4. Results

PART 1

Cloning of cDNAs encoding ABCB5β transporters into pDEST plasmids for expression in mammalian cells

ATP hydrolysis is required for the transport of substrates across membranes. To determine the activity and the preferred conformation of ABCB5β transporters (homodimer or heterodimer) to transport substrates, Gerard *et al*. prepared several constructs for transporters' expression in insect cells to carry out ATPase assays [38]. These constructs contain each interacting partners alone including ABCB5β, ABCB6 and ABCB9, and each interacting partner fused together with a linker to force the heterodimerization knowing: ABCB5β_P-gp linker_ABCB6, ABCB6_P-gp linker_ABCB5β, ABCB5β_P-gp linker_ABCB9 and ABCB9_P-gp linker_ABCB5β. The P-gp linker is derived from ABCB1 (also known as P-gp). Both possible orientations were addressed. ABCB5FL, the typical full ABC transporter was also cloned.

In this study, we wanted to further characterize these transporters in mammalian cells. As a first step, we generated constructs compatible with expression in mammalian cells. To facilitate the detection of these transporters, we used a vector with a GFP tag in the N-terminus region of the ABC transporters' cDNAs (pDEST53 plasmid). The cloning was performed using gateway technology as detailed in the specific aims and methods sections. In our case, a unique LR reaction was necessary to obtain the transfer of ABC transporter cDNA from a pDONR to a pDEST53 plasmid. After cloning, insertion of the sequence of interest in the pDEST53 plasmid was analyzed by PCR using the primers described in **Table 3**. ABCB5β, ABCB6 and ABCB9 amplicons were expected at a size around 2500 bp, while ABCB5FL and ABCB5β heterodimers were expected at a size around 5000 bp (**Figure 15**). Finally, plasmid DNA was assessed using Sanger sequencing.

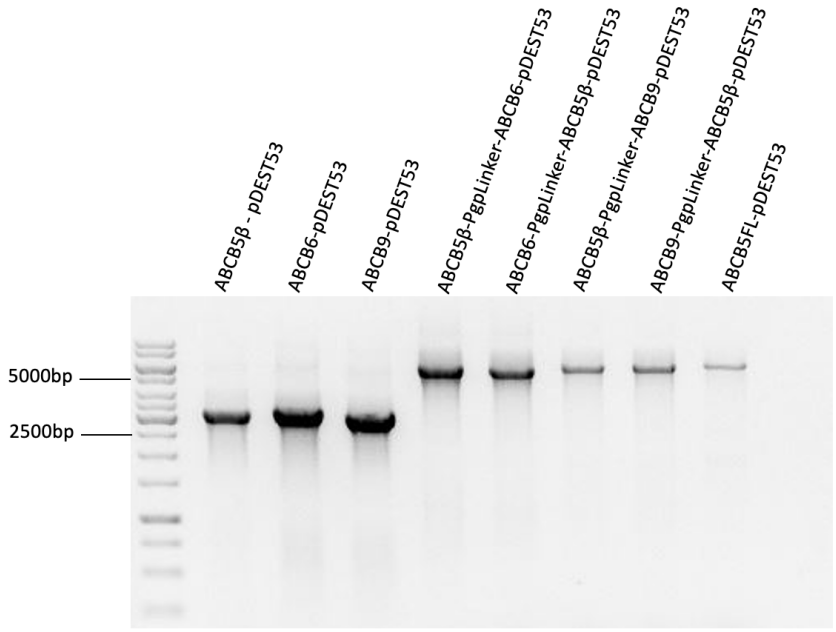


Figure 15 – PCR amplification of the eight genetic constructs with GFP tagged in their N-terminus. Gel PCR amplification of all constructions generated by gateway cloning, using primers displayed in table 3. 8µL of PCR product was loaded in 1% agarose gel.

First, we decided to work with HEK293T cells, which can be easily transfected. Following transfection with the different generated constructs, green fluorescence was analyzed using a fluorescent microscope. However, only detection of ABCB5β, ABCB5FL and ABCB9 was possible in HEK293T cells while nothing was seen for the other constructs visible in Figure 15. It is not excluded that the tag in N-terminus is not the preferred orientation for these constructs. To validate this hypothesis, three new constructs were generated but this time the tag was added in C terminus using a pDEST47 plasmid where the cDNA of interest is expressed upstream the GFP tag. Due to time constrain, only three constructs were generated: ABCB6_pDEST47, ABCB5β_P-gp linker_ABCB6_pDEST47 and ABCB9_P-gp linker_ ABCB5β_pDEST47. ABCB6 was chosen because of its orientation in lysosomal membrane. Its N terminus resides in the luminal side of lysosomes [42]. Therefore, lysosomal pH could interfere with GFP signal. ABCB5β_P-gp linker_ABCB6 and ABCB9_P-gp linker_ ABCB5β were chosen because their orientation showed the highest level of expression when expressed in high-five insect cells by Gerard et *al*. [38]. After the LR reaction, the insertion of the sequence of interest was analyzed by PCR using the primers described in **Table 3.** ABCB6 homodimer was expected at 2500bp and heterodimers at 5000bp (**Figure 16**).

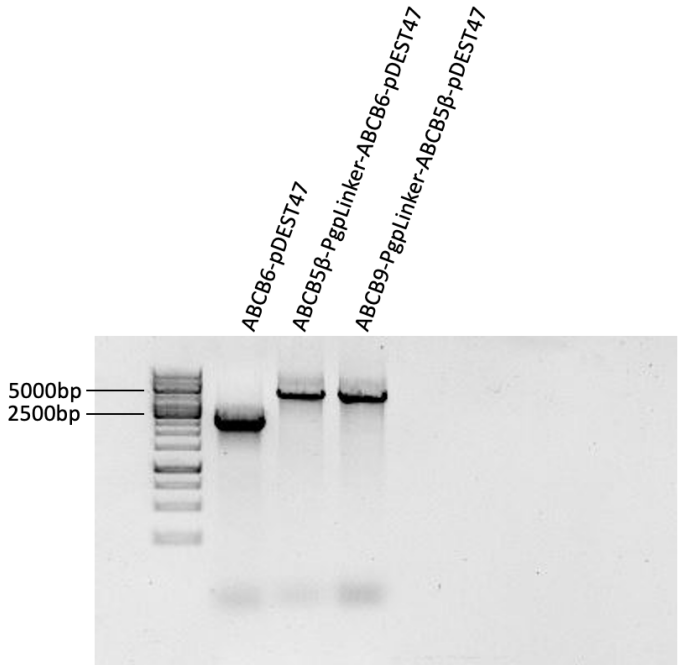


Figure 16 – PCR amplification of three constructs with a GFP tag upstream the corresponding cDNA in a pDEST47 plasmid. Gel PCR amplification of three constructions with the GFP tag in their C-terminus generated by gateway cloning, using proper primers displayed in table 2. 20µL of PCR product was loaded in 1% agarose gel.

These three generated constructs were transfected in HEK293T, however no signal could be detected. Because the studied heterodimers were, so far, only identified in melanoma, we decided to transfect these constructs in MelJuso, a melanoma cell line. Once again, no signal was obtained. Therefore, further experiments will only include ABCB5FL_pDEST53, ABCB5β_pDEST53 and ABCB9_pDEST53.

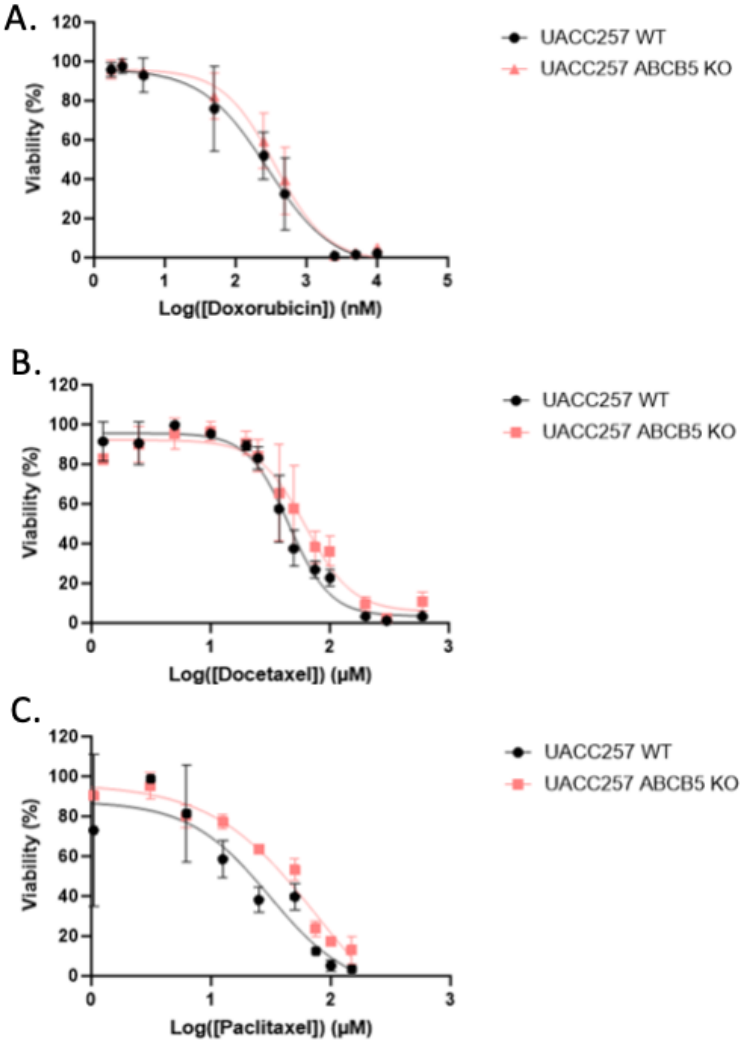
Role of ABCB5β and ABCB5FL in doxorubicin, docetaxel, and paclitaxel resistance

The first axis of this master thesis consists in studying the potential role of ABCB5β transporters in multidrug resistance, including its interaction partners ABCB6 and ABCB9. Preliminary data obtained by Louise Gerard using ATPase assay showed that ABCB5β/B6 and ABCB5β/B9 heterodimers do not seem to transport doxorubicin, while ABCB5FL does (data

not shown). However, the drawback of ATPase assays is the lack of sensitivity, which may lead to false negative results when changes in ATPase activity are too subtle. Furthermore, we might face decoupled transport when the transport of substrates is independent of ATPase activity. It was therefore decided to address the potential role of ABCB5β transporters in multidrug resistance using MTT assays. Since melanoma is intrinsically resistant to doxorubicin and because ABCB5FL confers resistance to anthracyclines, we decided to start with this drug [36,76]. Unfortunately, as mentioned above, transfection issues were faced for all the generated constructs except for ABCB9_pDEST53, ABCB5β_pDEST53 and ABCB5FL_pDEST53. Therefore, it was decided to perform MTT with these three constructs first.

After transfection of either ABCB9, ABCB5β or ABCB5FL in HEK293T, no changes in cell viability could be seen when treated with increased concentration of doxorubicin (data not shown). Since ABCB5FL implication in doxorubicin resistance has already been mentioned in the literature, it was decided to switch to a more relevant cell line: MelJuso [36]. In fact, it is not excluded that ABCB5FL is mislocalized in HEK293T cells since it is not constitutively expressed in this cell type. Moreover, HEK293T are lacking melanosomes which are responsible for melanin production and were proposed to be one of the organelles where ABCB5FL resides [77]. Therefore, MTT were performed in MelJuso. As seen in **Supplementary Figure 1**, once again, no change in viability could be observed for cells expressing either of the two isoforms of ABCB5 when compared to the wild type counterpart. The main difference noticed between this experiment design and the one presented in the literature is the transfection method. In the literature, cells were selected for several weeks after transfection to obtain a stable ABCB5FL overexpression [36]. Since we were working with transient transfection, ABCB5FL and ABCB5β expression remained limited in both HEK293T and MelJuso. Therefore, our objective was to find an alternative allowing us to study the role of ABCB5 in resistance while avoiding transient transfection. In the meantime, via a collaboration with Cyagen (Santa Clara, USA), we obtained a human ABCB5 knockout UACC257 cell line using the CRISPR/Cas9 technology (UACC257 ABCB5 KO). Exon 13 was removed which leads to the knockout of the three longest ABCB5 isoforms (ABCB5FL, ABCB5β, ABCB5α) in UACC257. Correct knockout of ABCB5 was validated by sequencing. In order to solve the issued we were facing with ABCB5 transfection, we decided to pursue the MTT with this cell line.

IC50 values of doxorubicin, docetaxel and paclitaxel were compared between wild type UACC257 and UACC257 ABCB5 KO. These drugs were selected because of evidence in the literature suggesting that ABCB5FL mediates resistance to these three compounds [36]. UACC257 cells were treated for 72 hours with doxorubicin and 24 hours with docetaxel and paclitaxel. A 24-hour treatment was used for docetaxel and paclitaxel based on preliminary data showing that UACC257 cells were becoming resistant to both anticancer agents after a 24-hour treatment, leading to increased cell viability for all the tested concentrations. MTT assays showed that UACC257 ABCB5 KO cell line is more resistant to doxorubicin, docetaxel, and paclitaxel than the wild type (**Figure 17A-B-C-D**).



D.

IC50	Doxorubicin	Docetaxel	Paclitaxel
UACC257 WT	280,6 nM	45,39 µM	30,87 µM
UACC257 ABCB5 KO	359,9 nM	62,38 µM	76,56 µM

Figure 17 – Measurement of viability of wild type UACC257 compared with UACC257 knock out for the ABCB5 gene, both treated with increased concentration of chemotherapeutics. Percent of viability of UACC257 WT was compared with UACC257 ABCB5 KO both treated with (A) doxorubicin (n=3), (B) docetaxel (n=3) and (C) paclitaxel (n=2). (D) Comparison of IC50 values, the concentrations at which 50% if the cells are killed by the chemotherapeutics added.

In contrast to the literature, ABCB5 does not mediate resistance to these three drugs in the current model used in this study [36]. Since one limitation of the MTT assay is the fact that different cell lines with different growth rates can produce false positive or false negative results, we decided to analyze the proliferation of the two tested cell lines with a proliferation assay. **Figure 18** shows that UACC257 ABCB5 KO cells proliferate at a much lower rate than UACC257 WT. If KO cells profiled faster than WT cells, this would explain why they were more resistant to the three treatments. A possible hypothesis concerning the puzzling MTT results, not explained by the proliferation test, could be that after the loss of ABCB5 gene, other ABC transporters sharing the same characteristics, might be overexpressed [78]. This was observed in Abcb5 mice, suppression of Abcb5 expression led to the overexpression of several

other Abc transporters, including Abcb1a [79]. ABCB1 is known to transport anthracyclines and taxanes, therefore, we hypothesized that its expression might be increased to compensate the loss of ABCB5 [15]. To investigate this, ABCB1 expression was analyzed by Western blot in UACC257 WT, UACC257 ABCB5 KO and UACC257 transfected with ABCB5FL or ABCB5β. A band at 170kDa, the expected size of ABCB1 was visible in all conditions (**Figure 19**). However, we were unable to quantify these bands as only one replicate was carried out, even though the intensity of ABCB1 appears to be the same in each conditions.

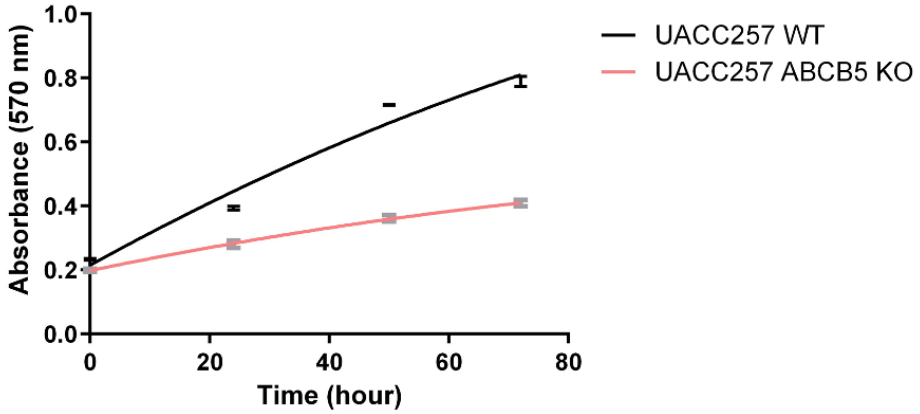


Figure 18 – Proliferation assay of UACC257 WT and UACC257 ABCB5 KO. Cells were plated in a 96-well plate and absorbance was read at 570nm every 24 hours (n=2). The black curve represents the proliferation of wild type UACC257 and the pink one is for UACC257 ABCB5 KO.

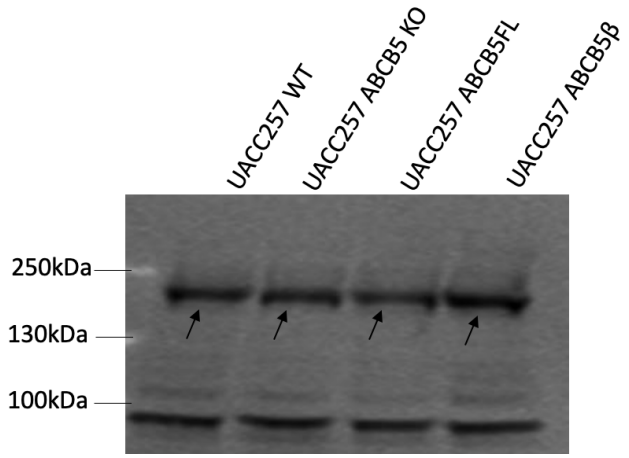


Figure 19 – Expression of ABCB1 in UACC257 WT, UACC257 ABCB5KO and UACC257 transfected with either ABCB5FL or ABCB5β. The expression of ABCB1 is seen in all conditions, were bands of interest are highlighted with black arrows.

PART 2

Expression of ABCB5FL, ABCB5FL E1181Q mutant and ABCB5β in insect cells

The second axis of this project aims to elucidate the pharmacological and pharmacokinetic properties of ABCB5 transporters. This requires studying the ATPase activity of ABCB5 transporters in different conditions using the state-of-the-art ATPase assay. To do so, ABC transporters must be expressed in insect cells.

Because membrane vesicles expressing ABCB5β were already produced by Gerard et *al.,* the same material was used in this project [38]. Therefore, only insect cell vesicles expressing ABCB5FL and a non-functional ABCB5FL E1181Q mutant as control were missing and had to be prepared. To start, Sf9 insect cells were transfected with bacmid encoding the corresponding cDNA (ABCB5FL or ABCB5FL E1181Q mutant). ABCB5FL E1181Q mutant carries a mutation at the position 1181, in the Walker B motif of its second NBD, where a glutamic acid was changed to a glutamine, rendering this transporter non-functional as it is unable to hydrolyze ATP. After transfection, baculoviruses were produced and secreted in the medium. Infection signs were monitored for 96 hours (**Figure 20**).

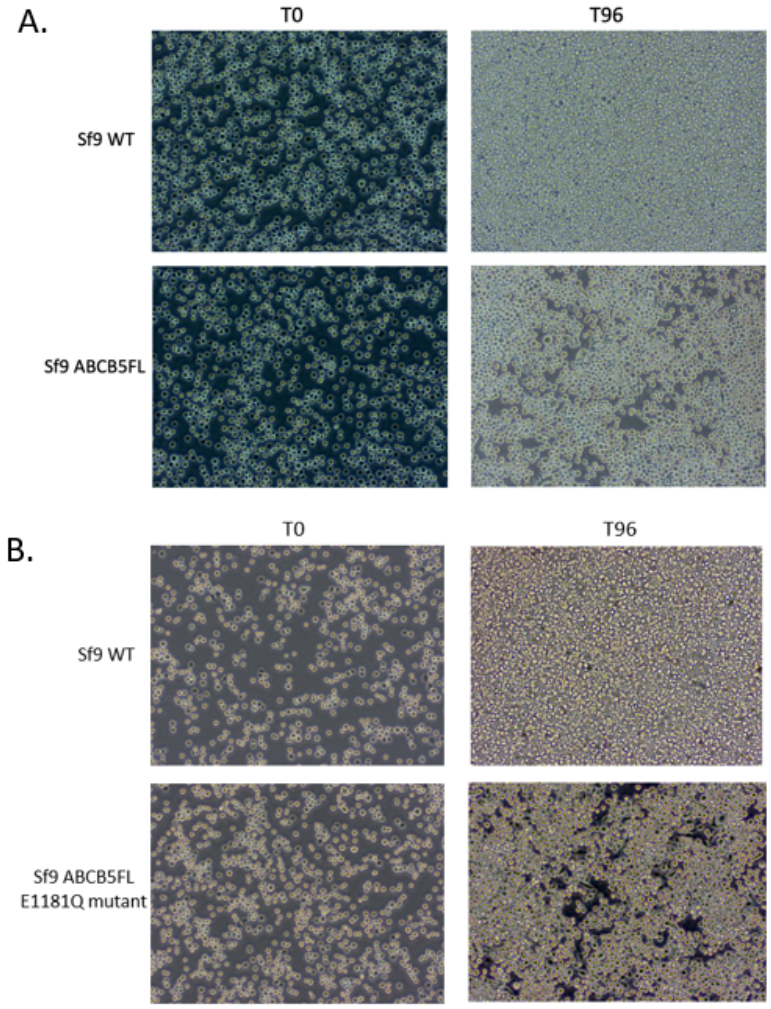


Figure 20 - Infection sign after transfection in Sf9 cells with bacmid encoding for (A) ABCB5FL or (B) ABCB5FL E1181Q mutant. Picture with a phase microscope at 10X magnification were performed on day zero, and 96-hour post transfection to monitor the infection signs (cell death, swelling, increased nuclei diameter, granular appearance, occlusions bodies, and so on).

Medium containing baculoviruses with the recombinant DNA of ABCB5FL or ABCB5FL E1181Q mutant were harvested and used to infect high-five insect cells with the corresponding baculoviruses at a MOI of 10. Cells were harvested 72 hours post infection. Expression of ABCB5FL and ABCB5 E1181Q mutant proteins were checked in high-five insect cells after membrane vesicles preparation by Coomassie staining (**Figure 21A**) and by western blot following SDS-Page (**Figure 21B**). A band around 140kDa was detected for ABCB5FL and ABCB5FL E1181Q mutant expressed in high-five membrane vesicles in both the Coomassie staining and the western blot, as well as a band around 90kDa for high-five membrane vesicles expressing ABCB5β. No band was detected in western blot from wild type high-five membrane vesicles.

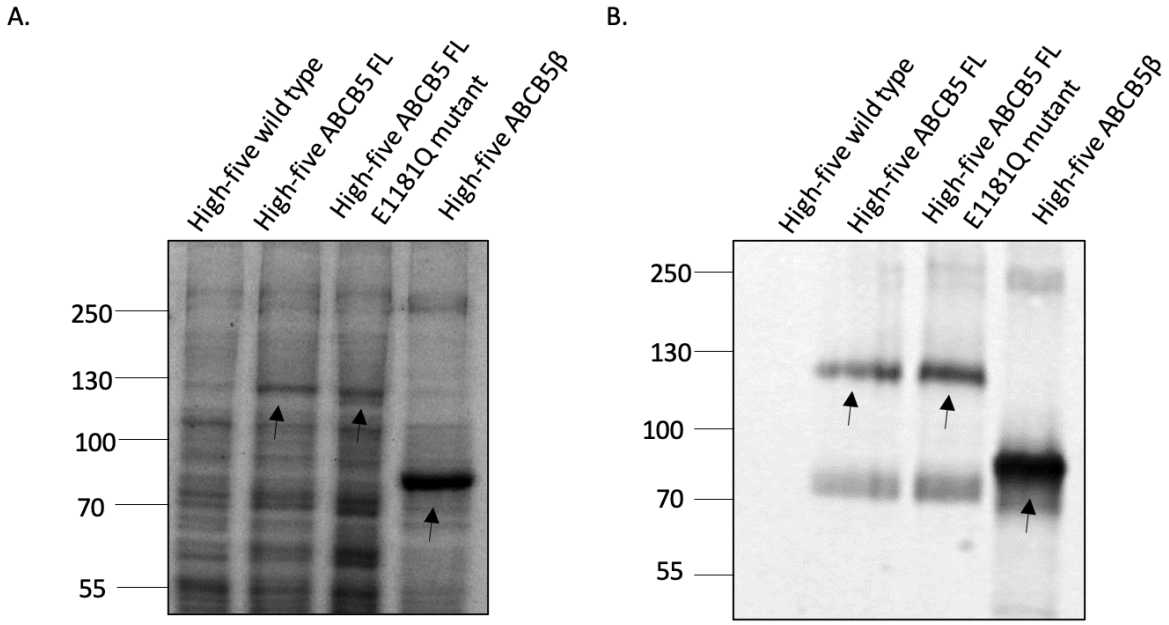


Figure 21 – Expression of ABCB5FL, ABCB5FL E1181Q mutant and ABCB5β in high-five insect cells. Total membranes vesicles prepared from high-five insect cells with baculovirus containing either ABCB5FL, ABCB5FL E1181Q mutant and ABCB5β have been detected on a (A) Coomassie-blue staining or (B) Western blotting with an anti-ABCB5 Rockland. Bands of interest are highlighted with dark arrows.

Pharmacokinetic characterization of ABCB5FL and ABCB5β

To better understand the difference between the two longest isoforms of ABCB5 (i.e ABCB5FL and ABCB5β) it was decided to further characterize both transporter ATPase activity when expressed in high-five insect cells. First, both transporters showed similar Michaelis-Menten kinetic (**Figure 22A-B**). Michaelis-Menten curves can determine the key kinetic parameters of a protein, such as the maximum speed (Vmax) at which an ABC transporter can hydrolyze ATP and the Michaelis constant (Km) that represent the concentration of ATP required to reach half of the Vmax of the ABC transporter. Km were 0.5324 ± 0.0709 mM for ABCB5FL and 0.6731 ± 0.0769 mM for ABCB5β, and Vmax were 25.67 ± 0.8759 nmol Pi/mg/min and 34.41 ± 1.069 nmol Pi/mg/min, respectively. For both transporters, negative controls were used where ATP could not be hydrolyzed due to a mutation in their second NBD (ABCB5β E736Q and ABCB5FL E1181Q, leading to a decrease of the ATPase activity; **Figure 22A and B**. Overall, these mutants demonstrate that the ATPase activity measured for ABCB5FL and ABCB5β is specific to the studied transporter and does not arise from contaminating ATPase activity.

Next, Gerard et *al*. have shown that ABCB5β ATPase activity is inhibited by beryllium fluoride (BeFx) but not vanadate [38]. We wanted to investigate if it was also the case for ABCB5FL. Since BeFx is composed of beryllium sulfate ($BeSO_4$) and sodium fluoride (NaF), these two compounds used to form BeFx, were also tested alone to determine whether they could also inhibit the ATPase activity of our transporters. BeFx and vanadate form stable complexes with ATP, interacting with its gamma-phosphate, preventing ATP-hydrolysis [71]. Inhibition was identical for both transporters **(Figure 22C-D-E**). BeFx represented with dark pink curves on **Figure 22C** and **D**, led to a 50% of inhibition for ABCB5FL and ABCB5β ATPase activity. On **Figure 22C** (dark pink curve), concentration of $BeSO_4$ was varying with fixed 2,5mM of NaF, which led to 44% inhibition for ABCB5FL and 48% inhibition for ABCB5β at 500µM. The same proportion of inhibition was reached when concentration of NaF was varying with fixed 200µM of $BeSO_4$ (**Figure 22D**). However, $BeSO_4$ alone led to 13% and 15% inhibition for ABCB5FL and ABCB5β respectively at 500µM (light pink curve on **Figure 22C**), like NaF alone led to 17% and 15% inhibition for ABCB5FL and ABCB5β (blue curves on **Figure 22D**). Vanadate led to 10% decrease in ATPase activity for ABCB5FL and 4% decrease for ABCB5β at 600µM which was the maximum inhibition reached (**Figure 22E**). Interestingly, ABCB5FL and ABCB5β display identical profiles when incubated with increased concentration of different known inhibitors of ABC transporters. Moreover, only the combination of NaF and $BeSO_4$ was able to inhibit the ATPase activity of both transporters.

Next, implication of $MgCl_2$ in ABCB5FL and ABCB5β ATPase activity was investigated. Both transporters' basal ATPase activity was analyzed in presence and absence of $MgCl_2$ (**Figure 22F**). The condition without $MgCl_2$ showed a decrease of more than 90% in ATPase activity (**Figure 22F**). It shows that ABCB5FL and ABCB5β ATPase activity is $MgCl_2$ dependent.

Then, ABCB5FL and ABCB5β thermostability was studied (**Figure 22G**). Because ABCB5β is a homodimer and ABCB5FL is already composed of two NBDs and two TMDs, we hypothesized that changes might be seen in the stability of these proteins at different temperatures. When their ATPase activity was analyzed at increasing temperatures, similar curves were obtained for the two transporters (**Figure 22G**). IT50 of ABCB5FL is 45.83°C and 46.2°C for ABCB5β. The IT50 indicates the temperature at which the transporter loses half of its ATPase activity.

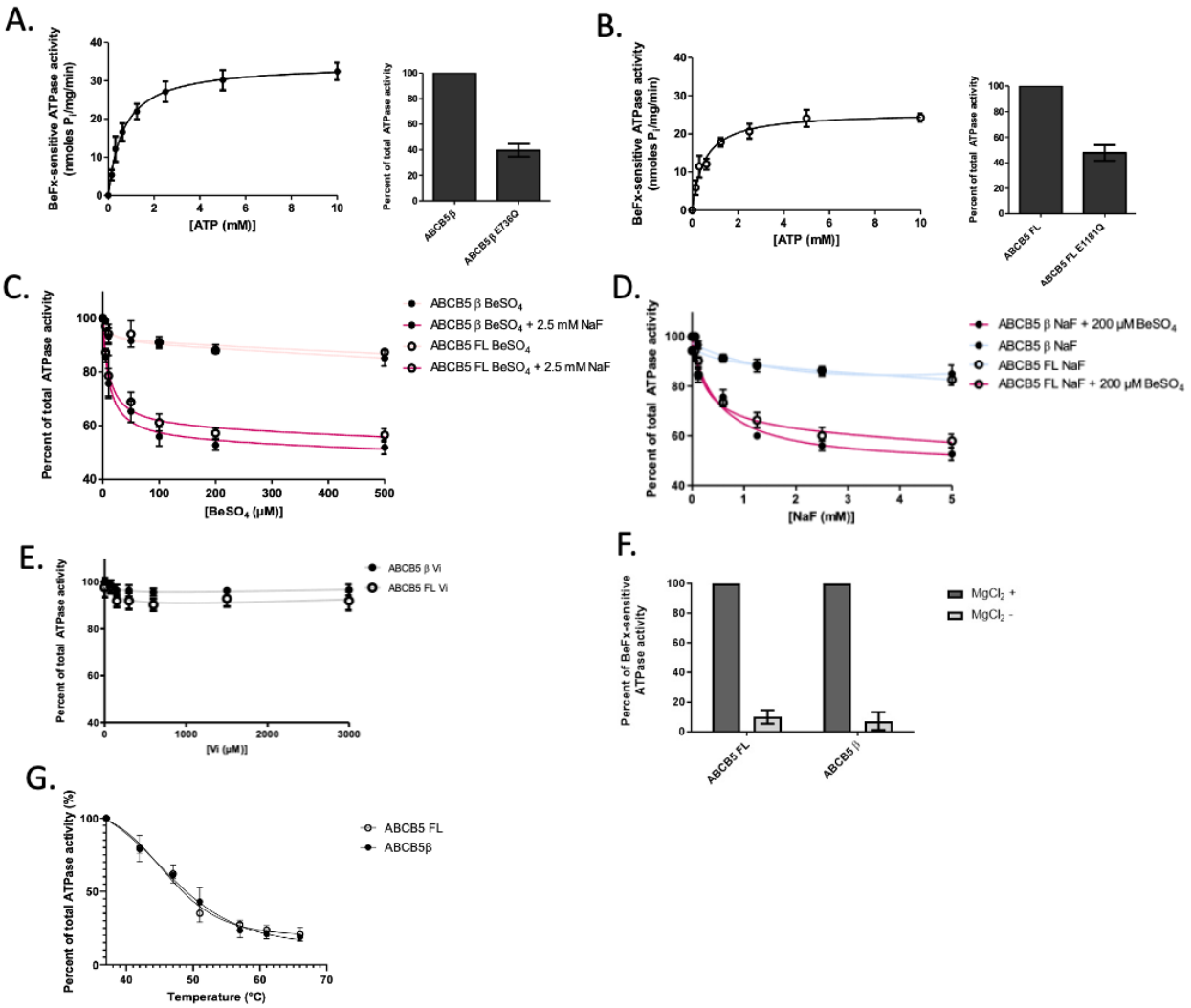
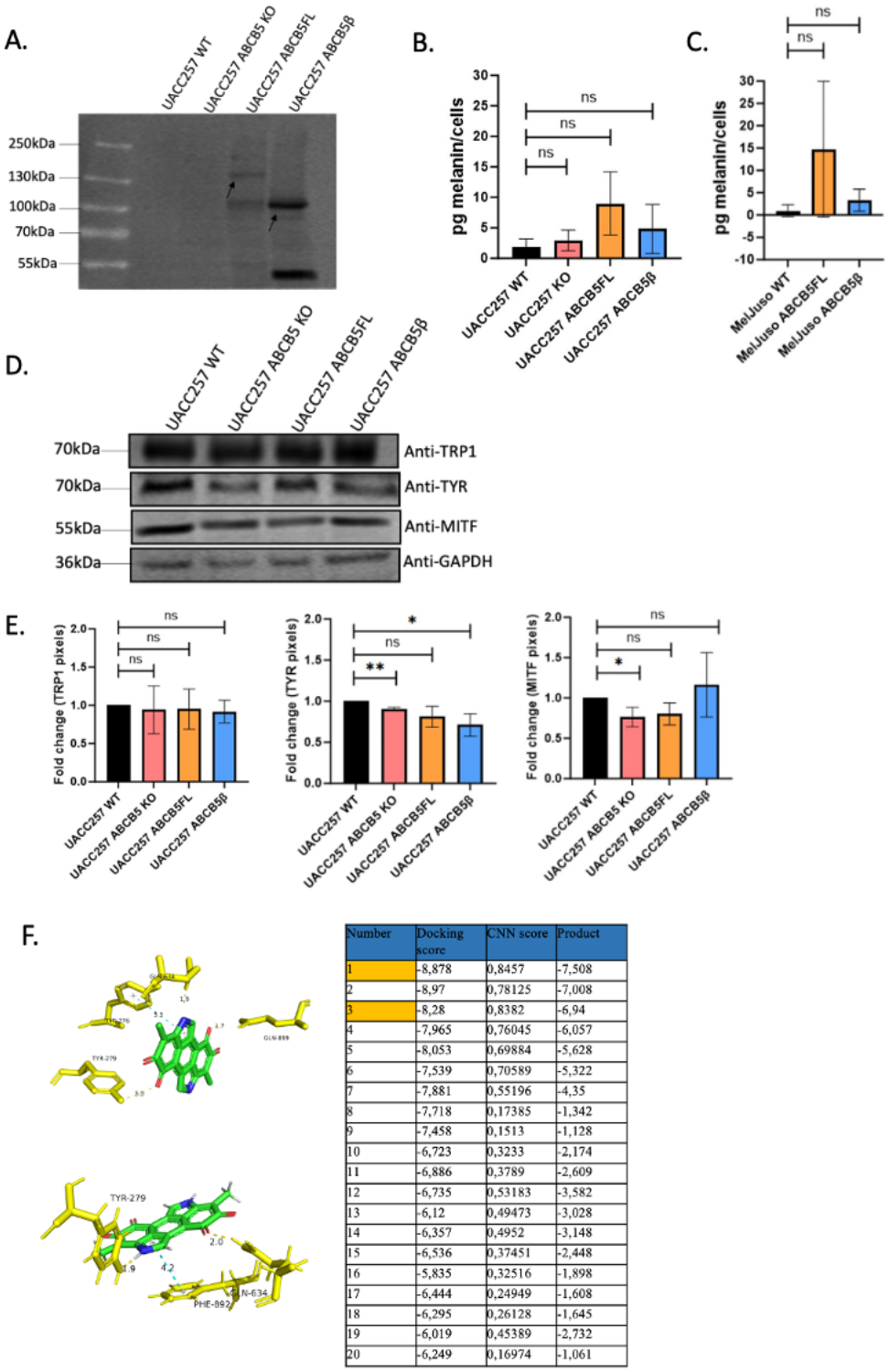


Figure 22 – Pharmacological characterization of ABCB5FL and ABCB5β. (**A**) Michaelis-Menten curve of ABCB5β transporter expressed in membrane vesicles using BeFx inhibitor (n=3). On the right, in absence of substrate, ABCB5β shows significant basal ATPase activity, while ABCB5β E736Q mutant cannot hydrolyze ATP because of a mutation in its second NBD. This construction was taken from the article published by Gerard et *al*. [38]. (**B**) Michaelis-Menten curve of ABCB5FL transporter expressed in membrane vesicles using BeFx inhibitor (n=3). On the right, in absence of substrate, ABCB5FL shows significant basal ATPase activity, while ABCB5FL E1181Q mutant cannot hydrolyze ATP because of a mutation in its second NBD. (**C-D-E**) Pharmacokinetic curve of ABCB5β and ABCB5FL in presence of increased concentration of (**C**) beryllium fluoride ($BeSO_4$) combined or not with 2,5mM of sodium fluoride (NaF), (**D**) sodium fluoride (NaF) combined or not with 200µM of sodium fluoride ($BeSO_4$) or (**E**) vanadate (Vi) (n=3 for each). (**F**) Influence of $MgCl_2$ on ABCB5FL and ABCB5β ATPase activity (n=3). (**G**) Thermostability of ABCB5FL and ABCB5β demonstrating their total percent ATPase activity from 37°C to 66°C (n=3).

PART 3

ABCB5FL and ABCB5β are not involved in melanin production

As the two isoforms of ABCB5 transporters are expressed in pigmented cells, it was hypothesized that they might be involved in melanin production [29,77]. Therefore, to determine if ABCB5FL or ABCB5β have an implication in melanin production, we dosed the amount of intracellular melanin in wild type UACC257, UACC257 ABCB5 KO and UACC257 overexpressing either ABCB5FL_GFP or ABCB5β_GFP (**Figure 23B**). Because MelJuso are usually transfected at a higher rate that UACC257, it was decided to also quantify the amount of intracellular melanin in wild type MelJuso and MelJuso transfected with ABCB5FL and ABCB5β (**Figure 23C**). As seen in **Figure 23A**, the expression of GFP-tagged ABCB5FL and ABCB5β in transfected UACC257 cells was checked using an anti-GFP antibody. A band around 170kDa for ABCB5FL was seen, as well as in 120kDa for ABCB5β, showing the correct expression of the transporters in UACC257. Then, the amount of intracellular melanin was quantified for all the conditions following the protocol detailed in the method section. Wild type UACC257 had an average of intracellular melanin of 1,8 pg/cells compared to 9 pg/cells for UACC257 overexpressing ABCB5FL (**Figure 23B**). More intracellular melanin was also contained in MelJuso cells transfected with ABCB5FL than in wild type cells (**Figure 23C**). However, the standard deviations of this test were very high, and the statistical analysis demonstrated no significant difference of intracellular melanin per cells between UACC257 WT and UACC257 ABCB5 KO or UACC257 overexpressing either ABCB5FL or ABCB5β (**Figure 23B**). Furthermore, no significant difference could be demonstrated between wild type MelJuso and MelJuso overexpressing either ABCB5FL or ABCB5β (**Figure 23C**).



Number	Docking score	CNN score	Product
1	-8,878	0,8457	-7,508
2	-8,97	0,78125	-7,008
3	-8,28	0,8382	-6,94
4	-7,965	0,76045	-6,057
5	-8,053	0,69884	-5,628
6	-7,539	0,70589	-5,322
7	-7,881	0,55196	-4,35
8	-7,718	0,17385	-1,342
9	-7,458	0,1513	-1,128
10	-6,723	0,3233	-2,174
11	-6,886	0,3789	-2,609
12	-6,735	0,53183	-3,582
13	-6,12	0,49473	-3,028
14	-6,357	0,4952	-3,148
15	-6,536	0,37451	-2,448
16	-5,835	0,32516	-1,898
17	-6,444	0,24949	-1,608
18	-6,295	0,26128	-1,645
19	-6,019	0,45389	-2,732
20	-6,249	0,16974	-1,061

Figure 23 –Investigation of the role of ABCB5FL and ABCB5β in melanin transport and production. (A) Expression of ABCB5FL_pDEST53 and ABCB5β_pDEST53 in UACC257 was checked using an anti-GFP antibody. ABCB5FL_pDEST53 appears at 170kDa and ABCB5β_pDEST53 at 120kDa. 50µg of protein was loaded on an 8% acrylamide gel. (B) Intracellular melanin dosage of wild type UACC257, UACC257 ABCB5 KO, UACC257 transfected with ABCB5FL or ABCB5β in 6-well plate. Statistics have been done using Student unpaired t-test on GraphPad Prism Software. ns represents not significant. (C) Intracellular melanin dosage of wild type MelJuso and MelJuso transfected with ABCB5FL or ABCB5β. Statistics have been done using Student unpaired t-test on GraphPad Prism Software. ns represents not significant. (D) TRP1, MITF, TYR and GAPDH expression in wild type UACC257 samples, UACC257 ABCB5 KO, and UACC257 overexpressing ABCB5FL

or ABCB5β were checked by SDS-Page followed by western blotting in an 8% polyacrylamide gel. 10µg of protein was loaded for the anti-TRP1 gel, while 50µg of proteins was loaded for the other antibodies. (n=3 for each) (E) Quantification of TRP1, TYR and MITF pixels normalized with GAPDH. Data are expressed as fold-change relative to UACC257 WT. Statistics have been done using Student unpaired t-test on GraphPad Prism Software. * P < 0,05, ** P < 0,005 and ns represents not significant. (F) Molecular docking analyzing the docking score and the convolutional neural network score (CNN score) of twenty combinations of ABCB5 amino acids with melanin, represented in green.

Then, to determine whether ABCB5FL and/or ABCB5β could be involved in melanin production, we compared the expression of three proteins involved in melanogenesis, which are tyrosinase (TYR), tyrosinase related protein 1 (TRP1) and microphthalmia-associated transcription factor (MITF) (**Figure 23D**). TYR and TRP1 are two tyrosinase, catalyzing different steps in melanin biosynthesis where MITF is a transcription factor involved in their synthesis [80]. No significant changes in expression were seen except for TYR and MITF expression in ABCB5 KO when compared to wild type and in TYR expression in ABCB5β when compared to wild type (**Figure 23E**). TYR and MITF expression were both lower in UACC257 ABCB5 KO and UACC257 ABCB5β compared to the wild type.

Based on this set of preliminary data, ABCB5FL and ABCB5β do not seem to influence melanin production in UACC257. In consequence, we wanted to determine if those transporters could be involved in the transport of melanin instead of its production. To do so, we performed molecular docking analysis on ABCB5L homology 3-D model (**Figure 23F**). This study aims to predict and analyze the molecular interactions between ABCB5FL and melanin, providing information about potential binding sites. First, ABCB5FL 3-D model was generated based on ABCB1 structure. Then, twenty different orientations were selected based on the best possible interactions between ABCB5FL and melanin. Among the twenty orientations, two were selected because they had the best docking score and convolutional neural network score (CNN score) (**Figure 23F**). The lower the docking score is, the stronger the interaction is, while the larger the CNN score is, the greater is the possibility of interaction. Amino acids Y276 and Y279, located in the first TMD of ABCB5FL and amino acids Q634, F892 and Q899 belonging to the second TMD of ABCB5FL were identified as potential interaction site for melanin (**Figure 23F**). The large CNN score indicates that ABCB5 could be involved in melanin transport. However, amino acids Y276 and Y279 are only present in ABCB5FL first TMD and not in ABCB5β. Therefore, based on these results, it is expected that ABCB5FL might be the only one involved in melanin transport since its first TMD is needed to interact with melanin. Similar results were obtained by Tangella et *al*., showing that doxorubicin, daunorubicin, paclitaxel, vincristine, camptothecin, etoposide, docetaxel, 5-FU and mitoxantrone binding pocket resides in ABCB5FL first and second TMD [24]. Therefore, ABCB5β might not be able to confer MDR because of missing residues necessary to form the binding pocket of the different substrates mentioned. To assess this hypothesis, three constructions were generated (**Figure 24**).

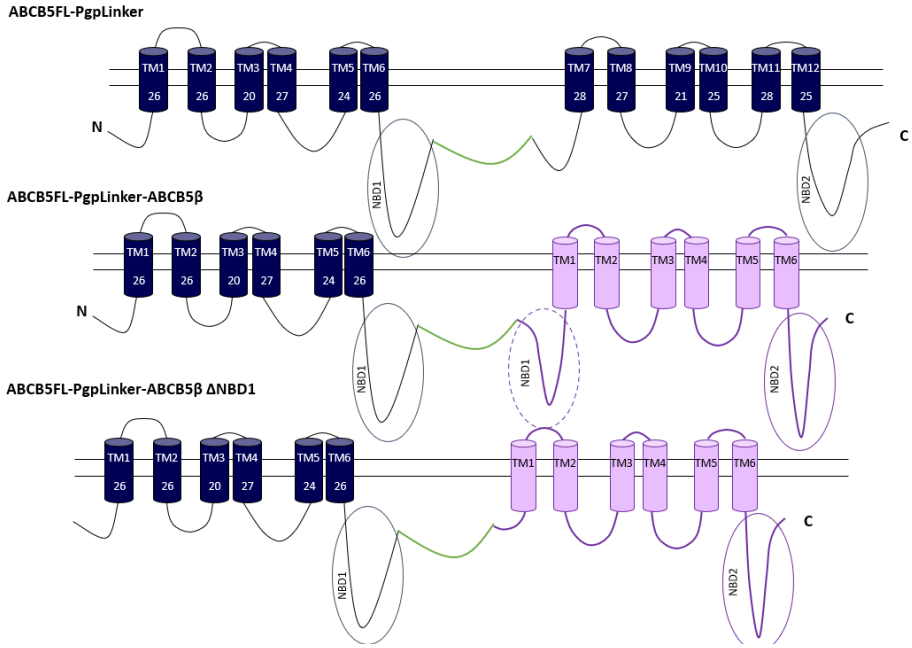


Figure 24 – 2D topology of the three new constructions generated in bacmids to understand how melanin can interact with ABCB5FL. On the top is represented ABCB5FL(half 1)_P-gp linker_ABCB5FL(half 2) where the sequence of ABCB5FL is interrupted with the P-gp linker it its middle. In the middle is ABCB5FL_Pgp linker_ ABCB5β where the Pgp linker is used to fuse the first TMD and NBD of ABCB5FL with ABCB5β. On the bottom is represented the 2D topology of the first part of ABCB5FL fused with ABCB5β without its first truncated NBD. The P-gp linker is represented in green, ABCB5L sequence is in blue while ABCB5β is in purple.

The first one is ABCB5FL transporter where we inserted the P-gp linker between its two TMDs to have ABCB5FL(half 1)_P-gp linker_ABCB5FL(half 2) (**Figure 24**). Adding the P-gp linker to this construct allows us to check that it does not affect the ability of ABCB5FL to transport melanin. The second one is the first half of ABCB5FL fused with ABCB5β using the P-gp linker to force the interaction: ABCB5FL(half 1)_P-gp linker_ABCB5β. Finally, ABCB5FL(half 1) was fused with ABCB5β without its truncated first NBD using the P-gp linker because its supplementary NBD might block the passage of substrates, namely ABCB5FL(half 1)_P-gp linker_ABCB5βΔNBD1. They were constructed in pDONR, transferred into pDEST plasmids using the gateway cloning technology, and finally ultra-competent bacteria (DH10bac) were used to transfer pDEST plasmids with the cDNA of the transporters into bacmids. The lac Z β-galactosidase method was used to determine which colonies have endured transposition between the pDEST and the bacmids inside the DH10bac. This technique relies on the detection of blue or white colonies (**Supplementary Figure 2**). White colonies are the one that have endured the transposition between our pDEST plasmid expressing the sequence of interest and the bacmids inside the bacteria, while blue ones did not express the sequence of interest. We have screened these colonies using PCR amplification followed by electrophoresis. If there has been no transposition with the DNA of interest, a band around 300bp will be seen, which is not the case for the three of them (**Figure 25**). M13 primers specifically target the 300bp bacmid sequence present in DH10bac.

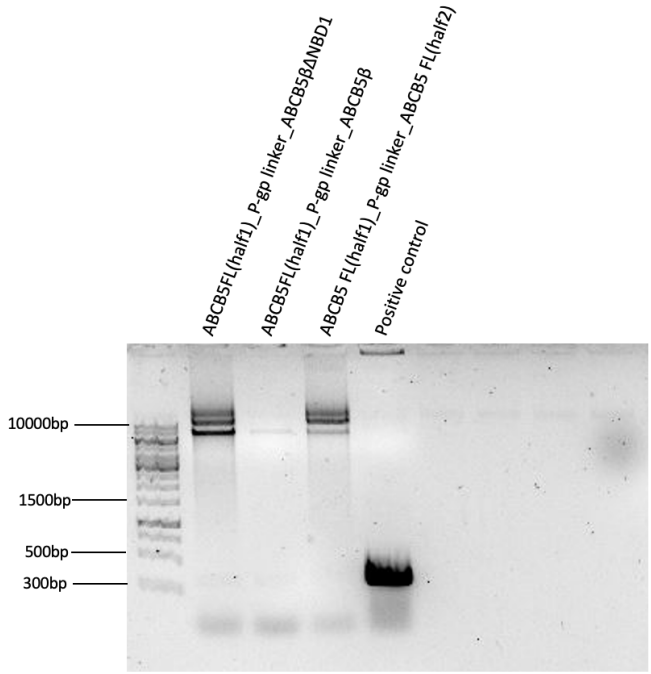


Figure 25 – PCR amplification of bacmids expressing ABCB5FL(half 1)_P-gp linker_ABCB5FL(half2), ABCB5FL(half 1)_P-gp linker_ ABCB5β and ABCB5FL(half 1)_P-gp linker_ABCB5βΔNBD1. M13 primers were used to detect any transposition between pDEST plasmid expressing our sequences of interest and the bacmids inside bacteria. If there has been no transposition, a band around 300bp must be seen, which is not the case for our three constructs. The positive control is a blue colony that has not endured transposition, where a band at 300bp is seen, which is size of the bacmid alone.

Bacmids expressing ABCB5FL(half 1)_P-gp linker_ABCB5FL(half2), ABCB5FL(half 1)_P-gp linker_ABCB5β and ABCB5FL(half 1)_P-gp linker_ABCB5βΔNBD1 were used to transfect Sf9 insect cells, to produce baculoviruses. Next, baculoviruses were used to infect high-five insect cells. Unfortunately, due to time constraints, this project stopped at the transfection of Sf9. Further experiments are needed to produce high-five insect cells expressing the three above-mentioned constructs. Later, membrane vesicles will be prepared, and ATPase assays will be performed in order to determine whether ABCB5FL first TMD is necessary for substrate transport.

5. Discussion and Perspectives

ABCB5 exists under several isoforms but only the two longest, ABCB5FL and ABCB5β, can function as transporters and could mediate MDR. In the literature, only few studies have specified which isoform was investigated. In consequence, there is a need to characterize the specific physiological function and implication in MDR of both isoforms separately. Recently, Gerard et *al*. showed that ABCB5β, a half transporter, heterodimerizes with ABCB6 and ABCB9 in melanoma [38]. Both heterodimers were shown to have a basal ATPase activity. However, none of the tested compounds (few vinka-alcaloids, anthracyclines and anti-metabolites) seemed to be potential substrates of these transporters using ATPase assays. However, ATPase assays lack of sensitivity could lead to false negative results. Therefore, we wanted to address the potential implication in MDR of ABCB5β homo- and heterodimers, and ABCB5FL using cytotoxic assays in mammalian cells. To study ABCB5FL, ABCB5β/B5β, ABCB5β/B6 and ABCB5β/B9 in mammalian cells, we prepared constructions suitable for expression in mammalian cells and with a GFP tag to facilitate detection of the protein of interest. To do so, constructs generated in Gerard et *al*. were cloned in a pDEST53 plasmid [38].

Only ABCB5FL_pDEST53, ABCB5β_pDEST53 and ABCB9_pDEST53 expression could be identified in HEK293T. Because transferring the cDNA from a pDEST53 (GFP located in N-terminal) to a pDEST47 (GFP located in C-terminal) did not lead to detection of GFP, we decided to pursue with ABCB5FL_pDEST53, ABCB5β_pDEST53 and ABCB9_pDEST53 only. In a MTT test, no change in IC50 could be observed for doxorubicin when cells were transfected with the three transporters of interest. However, ABCB5FL have been shown to mediate resistance to doxorubicin [36,76]. As HEK293T are lacking melanosomes, which could be one of the organelles where ABCB5FL resides, we performed new MTTs in MelJuso, a melanoma cell line, but again no changes in IC50 was observed [77]. To study ABCB5 implication in MDR without using transient transfection, we decided to use UACC257 ABCB5 KO obtained using the CRISPR/Cas9 technology by Cyagen. The MTT showed that when treated with doxorubicin, docetaxel, and paclitaxel, UACC257 ABCB5 KO had a slight increase in viability when compared to wild type UACC257. Interestingly, these results contradict those published in the literature [36,76]. The conflicting results may be explained by the fact that MTTs are not designed to compare different cell types and differences in terms of proliferation could lead to false positive results. Ghasemi et *al.* have compared all these parameters together and have reported that increasing the number of cells per well increases cell density, which in turn increases the metabolic activity, resulting in a higher read absorbance [81]. Moreover, more cells lead to increased survival after chemotherapeutic treatment. If the cells do not grow at the same rate, it is almost impossible to have the same number of cells through the 24 or 72 hours of incubation. Therefore, we performed a proliferation assay to determine if both cell lines were growing at the same rate or not. UACC257 ABCB5 KO proliferated more slowly than UACC257 WT. However, based on the results obtained with the MTT, we were expecting the opposite which would have explained the strong resistance of the UACC257 ABCB5 KO cell line. Maybe a compensation mechanism took place after the knockout of ABCB5 leading to chemoresistance. For example, ABCB1, also responsible for the transport of anthracyclines and taxanes, could be overexpressed in UACC257 ABCB5 KO to compensate for the loss of ABCB5 [15]. The occurrence of genetic compensation in reaction to gene knockout is a commonly observed phenomenon but the mechanisms behind are poorly understood [78]. Losing one gene is offset by the presence of another that shares similar functions, which partially or completely salvage the final result. For example, the loss of ABCC2 is compensated by ABCC3 due to their large

overlap of substrates specificity [82]. However, we did not observe the overexpression of ABCB1 in UACC257 ABCB5 KO compared with UACC257 WT. Nevertheless, it is important to keep in mind that only one replicate was performed and only the expression of ABCB1 was analyzed. Other ABC transporters have been shown to mediate resistance to doxorubicin, docetaxel, and paclitaxel and could be analyzed. For example, ABCC1, ABCC2, ABCC10 or ABCG2 [15,83]. In addition, the best solution would have been to carry out transport assays on membrane vesicles expressing either ABCB5FL or ABCB5β compared with wild type to determine if either doxorubicin, docetaxel, or paclitaxel could be one of their substrates. However, it would have required other membrane vesicles that the one we generated, using artificial liposomes and fluorescent substrates [84–86]. Gene-expressing liposomes give rise to greater expression of ABC transporter and lower background noise, which overall provide a greater sensibility. Another approach was proposed by Robinson et *al*. who developed a transport assay that employs mass spectrometry to detect unlabeled substrates loaded into liposomes [87]. However, these two methods are beyond the scope of this master thesis due to time constraints.

Next, we wanted to characterize the pharmacokinetic profiles of ABCB5FL and ABCB5β transporters due to the lack of information in the literature about these transporters. To do so, we used the membrane vesicles already prepared by Gerard et *al*. and produced membrane vesicles, from high five insect cells, expressing ABCB5FL and ABCB5FL E1191Q mutant [38]. First, the sequence of ABCB5FL and ABCB5FL E1181Q mutant were expressed in bacmids, and then transfected in Sf9 insect cells. Baculoviruses produced after transfection were amplified and titrated to infect high five insect cells at a MOI of 10. Next, several steps of ultracentrifugation were performed to prepare membrane vesicles expressing the transporters of interest. Michaelis Menten kinetics of ABCB5FL and ABCB5β expressed in membrane vesicles were similar (0.5324 ± 0.0709 mM and 0.6731 ± 0.0769 mM respectively). ABCB9 shows a similar Km value of 0.3 ± 0.06 mM, as for ABCB1 that ranges from 0.3 to 1.4 mM depending on experimental conditions [88]. EQ mutant of ABCB5FL and ABCB5β showed a significant decrease in ATPase activity showing that the ATPase activity seen for ABCB5FL and ABCB5β is specific to these transporters and does not arise from contaminating ATPase activity. Similar results were seen for ABCB6 E752Q mutant, harboring a mutation in its Walker B motif [89]. ABCB5FL and ABCB5β ATPase activity were not inhibited by vanadate but a 50% decrease was observed with BeFx. Interestingly, Chen et *al.* reported that ABCB2 and ABCB3 heterodimer is also less sensitive to vanadate compared to BeFx [90]. The same mechanism appears for ABCC6, but in this case vanadate could only inhibit the ATPase activity in presence of Ni^{+} [91]. This might be explained by the conformation adopted by each inhibitor because they inhibit different intermediates of the ATP cycle [71]. The MgADP.BeFx complex adopts a tetrahedral conformation around the Be atom, blocking it in a prehydrolytic conformation [70,92]. It therefore forms stable complexes that bound to NBDs to prevent ATP hydrolyzing [90]. The ADP.Mg^{2+}.Vi complex would have a bipyramidal trigonal geometry due to the covalent pentacovalent vanadate atom [70,71]. However, vanadate have been shown to inhibit other ABC transporters. In 1995, Ambudkar and colleagues showed that transport of vinblastine by ABCB1 expressed in proteoliposomes was completely blocked in the presence of 250µM vanadate [85,93]. Inhibition of ABCB5FL and ABCB5β ATPase activity by the two components forming BeFx, NaF and BeSO4, were similar to the one observed with vanadate, around 15%. Next, we showed that both transporters ATPase activity are $MgCl_2$ dependent, and they have a similar thermostability (45.83°C and 46.2°C for ABCB5FL and ABCB5β, respectively). Sauna et *al*., have conducted a similar study to perform the molecular pharmacology characterization of ABCC4 using ATPase assays on membrane vesicles derived from high five insect cells [94]. Similarly, they showed that ABCC4 ATPase activity is $MgCl_2$

dependent. Also, ABCB1 IT50 (44.4°C) determined by Lusvarghi et *al*. is similar to the one observed for our transporters of interest [95]. Overall, it shows that beside the difference in their structure, both transporters had identical pharmacological properties.

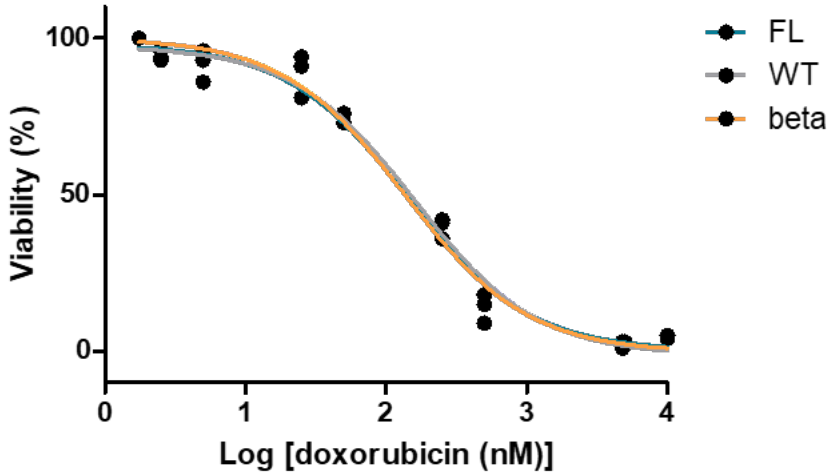
Finally, because the physiological function of ABCB5FL and ABCB5β has not been characterized yet, we decided to investigate their potential implication in melanin production. In the literature, it was hypothesized that ABCB5 transporters might be involved in melanin production as its two isoforms are overexpressed in pigmented cells [29,77]. To explore this further, we quantified the quantity of melanin produced in UACC257 WT, UACC257 ABCB5 KO and UACC257 transfected with ABCB5_GFP and ABCB5β_GFP. To compare our results with another pigmented melanoma cell line, we carried out melanin quantification of wild type MelJuso and MelJuso overexpressing either ABCB5FL or ABCB5β. No significant changes were detected in terms of pg of melanin per cells. Nevertheless, ABCB5FL overexpression in UACC257 and MelJuso showed increased concentration of melanin. The lack of significative results following statistical analysis could be explained by the high standard deviation obtained. Next, we quantified the expression of proteins involved in melanogenesis, TRP1, TYR and MITF [80,96]. Compared to wild type samples, both TYR and MITF expression was decreased in UACC257 ABCB5 KO. Moreover, TYR expression was decreased in UACC257 transfected with ABCB5β compared to UACC257 WT. This was surprising because if our hypothesis proved to be true, we should have seen an increase in the expression of proteins involved in melanogenesis in UACC257 transfected with ABCB5FL or ABCB5β and not a decrease as is in our first set of data. Even though MITF is a transcription factor that can bind to TYR and TRP1 promotors to regulate their expression, Fang et *al*. discovered that MITF has more strength to bind to TYR promotor than TRP1 promotor and therefore TYR is more highly expressed than TRP1 [97]. There was no direct correlation between MITF and TRP1 expression [97]. It could explain why the quantification of TRP1, TYR and MITF expression in each condition was not correlated. Interestingly, She et *al*. have performed the same study as we did with the ABCB6 transporter to analyze the effect of ABCB6 knockdown on melanogenesis [98]. They discovered that the knockdown of ABCB6 on two melanoma cell lines, MNT-1 and PIG1, led to a decrease of intracellular melanin level due to a downregulation of MITF (at the mRNA and protein level) and a downregulation of proteins involved in melanogenesis (TYR, TRP1 and TRP2). Bergam et *al*. also demonstrated that ABCB6 was necessary for early steps of melanogenesis regulation in MNT-1 cells [99]. Further experiments are needed to determine if ABCB5 could be involved in melanogenesis. Nevertheless, because ABCB5 could be involved in melanin transport and not in its production, we decided to perform a molecular docking analysis to determine if melanin could be one of the ABCB5 substrates. Molecular docking analysis indicate that ABCB5FL might be responsible for the binding of melanin, whereas ABCB5β may not. Amino acids Y276 and Y279, located on first ABCB5FL TMD were found to be the potential interaction site for melanin. Tangella et *al*. have also discovered important residues localized in first and second ABCB5FL TMDs responsible for the binding of some chemotherapeutics through molecular docking studies [24]. It has become a valuable tool for many applications and for example to discover new drug targets [100,101].

To validate if ABCB5FL transports melanin, ATPase assay must be performed. Because ABCB5FL first TMD seems to be necessary for the transport of substrates, we generated three new constructs to help us determine if the absence of this TMD in ABCB5β is the reason why this transporter is not associated with MDR in the literature [37]. The first one is the whole structure of ABCB5FL supplemented with the P-gp linker to determine if it does not affect the ability of the transporter to hydrolyze ATP and transport melanin. Then, we added the first TMD of ABCB5FL to ABCB5β using the P-gp linker to determine whether the second

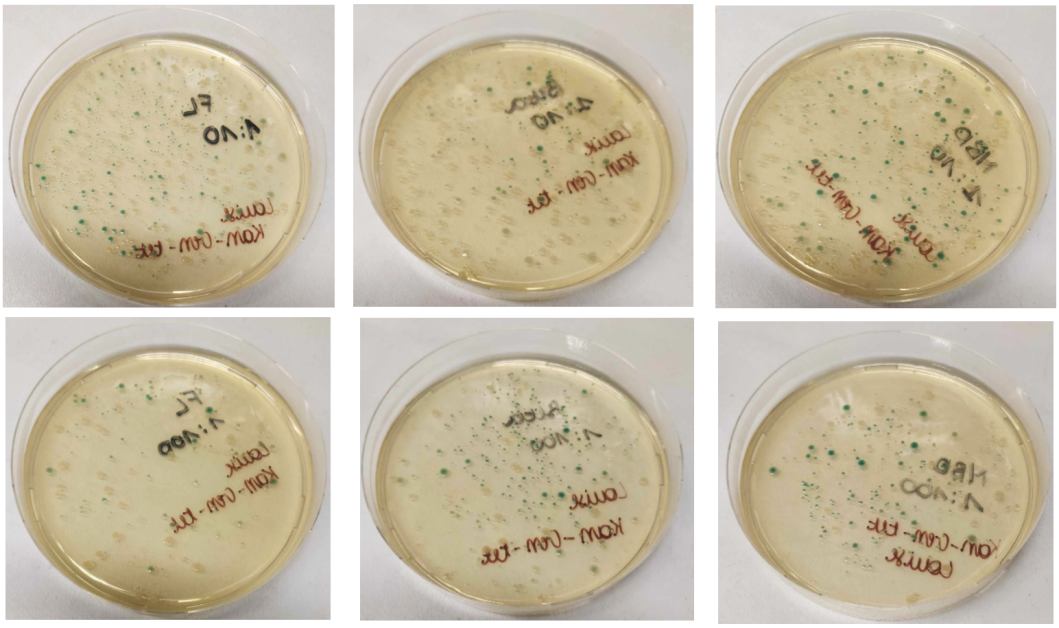
ABCB5FL TMD is necessary to transport melanin. The last one is ABCB5β without its truncated first NBD added to ABCB5FL first TMD to analyze whether the truncated NBD of ABCB5β can block substrate passage.

Overall, we generated constructs of ABCB5FL and ABCB5β homodimers and heterodimers for their expression in mammalian cells. Only ABCB9, ABCB5FL and ABCB5β could be transfected. In the meantime, UACC257 ABCB5 KO were obtained, and we decided to include this cell line in the study that we performed. The MTT assays showed contradictory results that could potentially be explained by compensatory mechanisms. Next, ABCB5FL and ABCB5β had similar pharmacokinetic profiles. Finally, further experiments are needed to determine whether ABCB5FL and ABCB5β might be involved in melanin transport and/or production. Moreover, we proposed that ABCB5β is not involved in anti-cancer agent transport because this transporter is lacking several residues that were shown to be implicated in substrate transport in ABCB5FL. To validate this hypothesis, three constructs were prepared in high five insect cells membrane vesicles and will be used to perform ATPase assays.

6. Supplementary section



Supplementary Figure 1 - Measurement of viability of wild type and transfected MelJuso cells treated with doxorubicin. Wild type MelJuso and MelJuso transfected with ABCB5FL or ABCB5β, both with a GFP tag in their N-terminus were treated 72 hours with increased concentration of doxorubicin. FL stands for MelJuso transfected with ABCB5FL, WT for MelJuso wild type and beta for MelJuso transfected with ABCB5β.



Supplementary Figure 2 – Generation of ABCB5FL(half 1)_P-gp linker_ABCB5FL(half 2), ABCB5FL(half 1)_P-gp linker_ ABCB5β and ABCB5FL(half 1)_P-gp linker_ABCB5βΔNBD1 expressed in bacmids. The DNA of interest was added in bacmids following the LacZ method where white colonies are the plasmids that have been transposed. Up are showed 1/10 dilution and down is 1/100 dilution of plasmids with DNA of interest. On the left is ABCB5FL(half 1)_P-gp linker_ABCB5FL(half2), on the middle is ABCB5FL(half 1)_P-gp linker_ ABCB5β and on the right is ABCB5FL(half 1)_P-gp linker_ABCB5βΔNBD1. After having grown up on kanamycin plates, white colonies have been screened on PCR amplification following by electrophoresis on a 1% agarose gel.

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