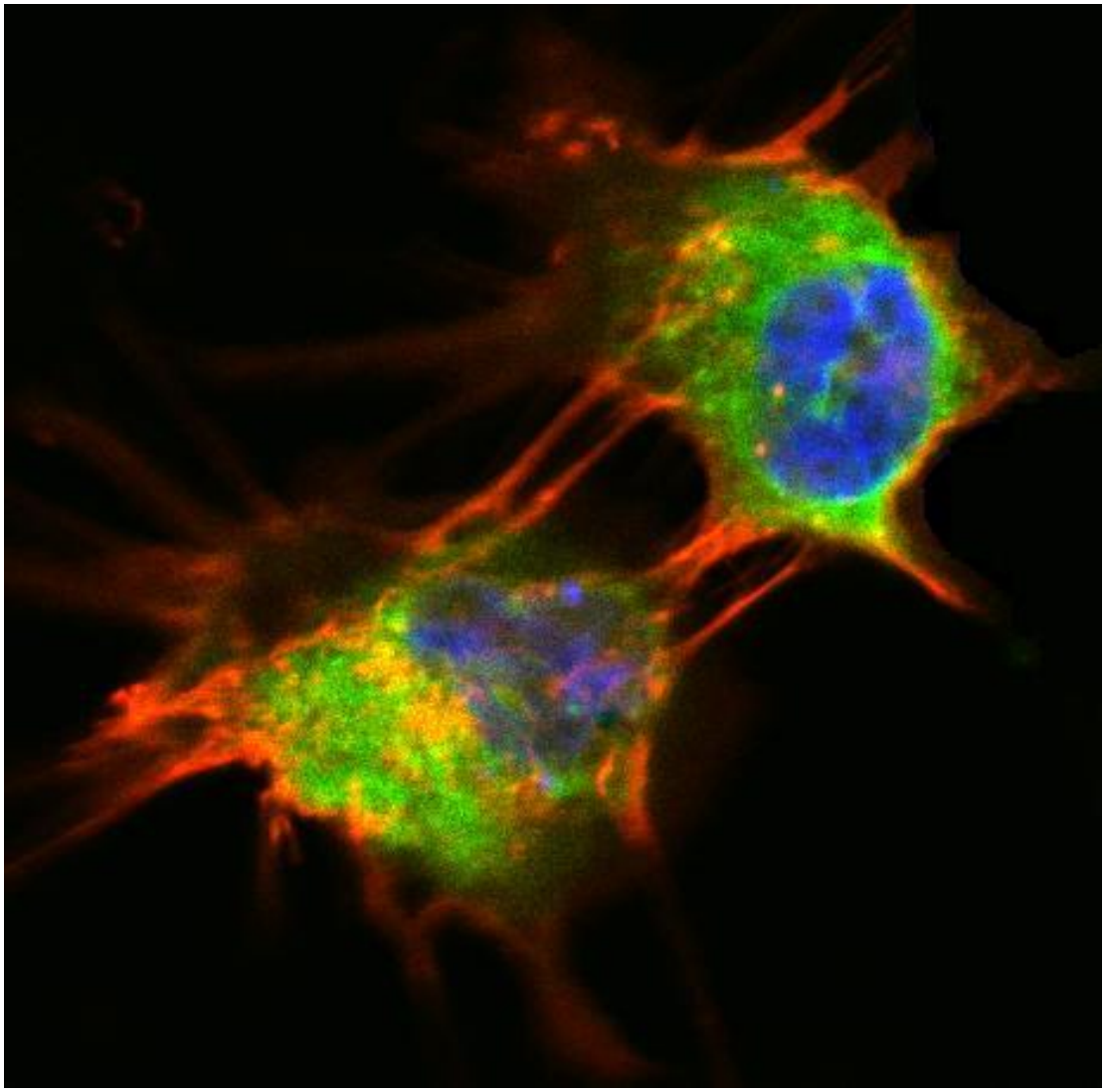
CFTR correctors enhance ATP8B1 I661T plasma membrane expression

A novel treatment for ATP8B1 deficiency?



M.A.M. Lohuis

Research project supervisor: Dr. S. van de Graaf

Cover picture: ATP8B1, a protein expressed at the cell surface and involved in a form of intrahepatic cholestasis, needs co-expression with protein CDC50A to reach the plasma membrane. Cells were cotransfected with HA-ATP8B1 and CDC50A-V5. HA-ATP8B1 was visualized with primary mouse-α-HA and secondary goat-anti-mouse Alexa-568(red). CDC50A-V5 was visualized with mouse-anti-FITC-V5 (green). Nucleus is stained with DAPI (blue). ATP8B1 is detected at the plasma membrane, partially co-localized with CDC50A. CDC50A is detected intracellular and slightly at the plasma membrane.

July 5, 2011

CONTENT

ABSTRACT

Mutations in the *ATP8B1* gene can cause benign recurrent intrahepatic cholestasis 1 (BRIC1) or progressive familial intrahepatic cholestasis 1 (PFIC1), commonly known as ATP8B1 deficiency. ATP8B1 is an aminophospholipid flippase, maintaining membrane asymmetry. In ATP8B1 deficiency the activity of the bile salt export pump (BSEP) which pumps bile salts out of the hepatocytes into the bile duct is decreased, leading to accumulation of bile salts and cell damage. However, the mechanism by which ATP8B1 defects causes cholestasis is unknown. Involvement of the nuclear receptor FXR or reduced membrane stability and consequent decreased BSEP activity have been suggested. Proper ATP8B1 folding and association with CDC50A is required for ATP8B1 to exit the ER and traffic to the plasma membrane. It was recently demonstrated that ATP8B1 mutations often result in protein misfolding and subsequent ER retention and protein degradation. Molecular chaperones facilitate protein folding and trafficking. The cystic fibrosis conductance regulator (CFTR) is frequently mutated causing cystic fibrosis due to misfolding of this chloride channel. Several pharmacological chaperones enhance CFTR plasma membrane expression. Therefore, the effect of three of these chaperones was tested on mutant ATP8B1 (I661T) expression. Corrector C1 showed the best result and enhanced total and cell surface ATP8B1 I661T expression by 25% and 90% respectively. This suggests that C1 particularly enhances trafficking of ATP8B1 I661T to the plasma membrane. Pharmacological treatment with this chaperone might be an effective therapeutic treatment to relieve the symptoms of BRIC1 patients that often have an I661T mutation.

Abbreviations:

ATP8B1, P-type ATPase member 8B1; BD, biliary diversion; BRIC, benign recurrent intrahepatic cholestasis; BSEP, bile salt export pump; CDCA, chenodeoxycholic acid; CF, cystic fibrosis; ER, endoplasmic reticulum; FXR, Farnesoid X receptor; GGT, gamma-glutamyl transferase; LT, liver transplantation; NB, not biotinylated; NT, not transfected, 4-PBA, 4-phenyl butyric acid; PC, phosphatidylcholine, PFIC, progressive familial intrahepatic cholestasis; PS, phosphatidylserine; TC, taurocholate; TfR, transferrin receptor;

INTRODUCTION

Benign recurrent intrahepatic cholestasis and Progressive familial intrahepatic cholestasis (BRIC and PFIC) are serious conditions leading to episodes of severe pruritus and jaundice or liver failure and death. In type 1 BRIC and PFIC these conditions are usually caused by mutations in ATP8B1, therefore commonly called ATP8B1 deficiency[1-6]. ATP8B1 is a P4-type ATPase, which functions as a putative aminophospholipid flippase thereby transferring aminophospholipids from the outer to the inner leaflet of the plasma membrane. This results in asymmetry of the membrane[1, 4, 6-8]. Furthermore the bile salt export pump has a decreased activity, leading to accumulation of bile salts in the hepatocytes resulting in cholestasis[5, 9]. Exactly how ATP8B1 deficiency leads to cholestasis is unknown although there are some hypotheses.

Unlike the proteins mutated in other types of BRIC/PFIC ATP8B1 is not only expressed in the liver but also in other tissues such as the intestine, stomach and pancreas[8]. Common treatment does not ameliorate extrahepatic symptoms and is therefore not sufficient[5]. It might therefore be interesting to search for new therapies.

Mutations in *ATP8B1* often lead to misfolding and subsequent degradation of the protein.[7] Mutant ATP8B1 rarely reaches the plasma membrane while it often still has some residual activity.[10, 11] Molecular chaperones are known to facilitate protein folding and trafficking. Several pharmacological chaperones, also known as correctors, have been discovered that improve the protein folding and trafficking of the cystic fibrosis conductance regulator (CFTR) which is affected in patients with cystic fibrosis. The question of this research is:

Can CFTR correctors also increase plasma membrane expression of ATP8B1 I661T, a mutation occurring in many BRIC1 patients?

This study starts out with an overview of the present literature on ATP8B1 deficiency (see Literature analysis). To answer the question relating to the effect of pharmacological chaperones on ATP8B1 expression, we first looked at wild-type ATP8B1 and ATP8B1 I661T expression when co-expressed with CDC50A, a protein known to be necessary for ER exit of ATP8B1. Then we added three different pharmacological chaperones to cells expressing the ATP8B1 I661T mutant and studied the effect with a biotinylation essay and western blotting.

LITERATURE ANALYSIS

1. Bile formation and transport in canaliculi

Bile has several functions. First, it is necessary to dispose endobiotics and xenobiotics. Second, it dissolves lipids and fat-soluble vitamins from the food so it can be absorbed. Third, it regulates cholesterol homeostasis[12]. Bile consists of three main components: phospholipids, cholesterol and bile salts. Hepatocytes synthesize bile salts from cholesterol and secrete them across the canalicular membrane. In the bile duct the increased osmotic gradient caused by the secreted bile salts attracts water and solutes[12]. The amphiphatic nature of bile salts renders them cytotoxic at high concentrations[2, 5, 12, 13]. The concentration of free bile salts is drastically reduced by the presence of other constituents of bile extracted by bile salts; phospholipids and cholesterol. Together they form mixed micelles. The effect is a protection of the apical membrane against the toxic effects of bile salts[2, 5, 12]. Phospholipids also increase the solubility of cholesterol[2]. When only cholesterol is present in the canalicular lumen this will result in precipitations and eventually cholelithiasis. From the bile duct the bile is transported to the small intestine where it can fulfill its function. Then bile salts are absorbed in the intestine via the apical sodium bile acid transporter (ASBT) and the organic solute transporter alpha/beta heterodimer. Via the portal vein bile acids are transported to the liver where they are taken up by the hepatocytes[1].

Several transporters play a role in hepatocyte bile salt homeostasis. Bile salts are taken up from the portal blood by a basolateral hepatocyte transporter, the sodium-taurocholate cotransporting polypeptide (NTCP)[14]. Secretion of bile salts from the hepatocytes into the canaliculum is conducted by the Bile Salt Export Pump (BSEP). BSEP is liver specific and localized in the hepatocyte canalicular membrane where it transports bile salts to the biliary canaliculi against a high concentration gradient[2, 5]. The protein is encoded by the *ABCB11* gene. In situations of high intracellular bile salt concentrations Mrp4 is a transporter that exports bile salts out of the hepatocytes into the blood. The kidney can efficiently excrete bile salts from the blood into the urine[14]. Phosphatidylcholines, the main phospholipids in bile, are transported from the inner to the outer leaflet by the ABCB4 transporter, also known as a phosphatidylcholine (PC) floppase. In the outer leaflet it can be excreted into bile[2]. The *ABCB4* gene encodes a phospholipid translocase that is predominantly expressed in the canalicular membrane of hepatocytes[2, 5]. Another constituent of bile, cholesterol, can be extracted from the membrane by bile salts but the canalicular membrane of the hepatocytes also contains a cholesterol transporter, the ABCG5/ABCG8 heterodimer[2].

FXR

Several mechanisms exist to ensure that bile salts can exert their physiological role, while cytotoxic accumulation of bile salts is prevented. Bile salts are endogenous ligands for the Farnesoid X receptor (FXR), exemplified by chenodeoxycholic acid (CDCA), one of the main bile salts in human[2, 15]. FXR is highly expressed in the liver and intestine where it regulates bile salt pool size and intracellular bile salt concentrations by sensing bile salts and controlling gene transcription of specific bile salt transporters and key enzymes involved in bile salt synthesis and detoxification.

FXR is predominantly expressed in the liver, kidney and intestine and is known to be involved in the regulation of expression of genes involved in cholesterol homeostasis[2, 16]. Excess cholesterol is converted to bile salts. A high cholesterol/bile salt ratio in the liver increases expression of the rate-

limiting enzyme level in bile salt synthesis: cytochrome P450 cholesterol 7α-hydroxylase (CYP7A1)[14]. Cholesterol catabolism and *CYP7A1* repression is regulated by nuclear receptors, including FXR. A direct transcriptional target of bile salts and FXR is the orphan nuclear receptor small heterodimer partner (SHP). SHP can bind the liver receptor homolog-1 (LRH-1) to block it from stimulating the *CYP7A1* promoter. SHP thereby represses CYP7A1 expression. In this way bile salt levels are linked to their own synthesis.
FXR null mice show an alteration in gene expression of genes involved in bile salt synthesis, transport and secretion and a significant increase in serum bile salt levels. BSEP/ABCB11 expression is 20-fold decreased and CYP7A1 and CYP8B1, two bile salt synthesis enzymes that are normally repressed when bile salts bind to FXR are not repressed in *FXR* null mice fed a diet containing 1% cholic acid. This confirms that bile salts are a natural ligand for FXR and that FXR is a key regulator of bile salt homeostasis[14].

2. BRIC/PFIC

Three BRIC/PFIC types

In patients with benign recurrent intrahepatic cholestasis (BRIC) or progressive familial intrahepatic cholestasis (PFIC) there is a defect in canalicular secretion of bile salts. The disease is caused by genetic defects and expresses in different ways: episodically and chronically[2, 5]. Benign recurrent intrahepatic cholestasis (BRIC) is the episodic and autosomal recessive form[1, 3]. The cholestasis attacks in BRIC patients resolve spontaneously, leaving no signs of scar damage to the liver[1, 3, 17]. Between the episodes there are no symptoms indicating cholestasis[17, 18]. However, BRIC sometimes proceeds into progressive familial intrahepatic cholestasis (PFIC), the chronic form which is also an autosomal recessive disease[1, 4-6, 18]. Chronic cholestasis manifests early in infancy and causes hepatic fibrosis and end-stage liver disease and eventually this can result in liver failure and death[1, 3]. BRIC/PFIC is now seen as a continuous spectrum of diseases.

There are three types of BRIC/PFIC. PFIC3 or ABCB4 deficiency is caused by mutations in the *ABCB4* gene, also known as *MDR3*, localized on the locus 7q21[2]. As mentioned above it encodes a phosphatidylcholine floppase. Biomedical parameters in ABCB4 deficiency and most other cholestatic diseases show a high gamma-glutamyl transferase (GGT) level, unlike PFIC1 and 2, where these parameters are normal to low. PFIC2 is caused by mutations in the *ABCB11* gene, localized on locus 2q24[19]. *ABCB11* encodes the ABCB11 protein, also known as the Bile Salt Export Pump (BSEP). PFIC1 is caused by mutations in the *ATP8B1* gene and therefore the disease is also termed ATP8B1 deficiency[1-6]. *ATP8B1* is located on locus 18q21 and the function of the ATP8B1 protein is still speculative[1, 4, 6].

ATP8B1 is a type 4 P-type ATPase[1-3, 5]. Type 4 P-type ATPases are believed to function as an aminophospholipid flippase[1, 4, 6, 8] that transports aminophospholipids from the outer to the inner leaflet of the plasma membrane[7, 20]. This results in an asymmetry and makes the membrane stable. ATP8B1 is a putative flippase for phosphatidylserine[12]. ATP8B1 is abundantly expressed in the bladder, stomach and intestine and to lesser extent in the liver and pancreas[8]. The transporter is expressed at the apical membrane of epithelial cells, including the canalicular membrane of hepatocytes[3, 5].

In ABCB4 deficiency the composition of the bile is altered: less PC is extracted from the membrane. The mixed micelles that are normally formed in the bile ducts now lack phosphatidylcholine increasing the toxicity of the bile salts. The high concentration of bile salts in the bile duct damages cellular membranes and thus causes injury and inflammation to the cholangiocytes, the cells lining the bile duct[5]. GGT is normally situated in the canalicular membrane. The damaging effect of the high bile salt concentration in the canalicular lumen extracts the membrane bound GGT and is thus the cause of the high GGT level in ABCB4 deficiency. Furthermore, the low phospholipid level reduces the solubility of cholesterols which can cause crystallization and consequently obstruction of small bile ducts: cholelithiasis[2].
In ABCB11 deficiency, BSEP-mediated secretion of bile salts into the canalicular lumen is affected. When the bile salts are not transported to the canaliculi there is no bile flow and the bile salts accumulate in the hepatocytes causing severe liver damage[2]. ABCB11 deficiency presents with pruritis and jaundice[1, 17]. Fat malabsorption due to a limited amount of bile in the intestine can lead to fat-soluble vitamin deficiencies such as predisposition to bleeding (vitamin K), osteomalacia/osteoporosis (vitamin D), neuromuscular abnormalities (vitamin E) and weight-loss[5]. Another appearance is a high risk of developing hepatobiliary malignancy in which the mechanism of carcinogenesis remains unknown. Further cholelithiasis occurs in 33% patients with ABCB11 deficiency. The cholelithiasis is probably due to the low bile salt concentration in the canaliculi because of the malfunction of BSEP, resulting in a relatively high concentration of cholesterol. ABCB11 deficiency starts from childhood and without treatment it will result in liver cirrhosis and eventually in liver failure[2, 5].

Unlike BSEP, ATP8B1 is not solely expressed in the liver. ATP8B1 deficiency therefore has the same symptoms as ABCB11 deficiency but it also presents with some extrahepatic symptoms, including pancreatitis, hearing loss, chronic respiratory problems and an elevated sweat electrolyte concentration[12, 17, 21]. The latter is similar to patients with cystic fibrosis who have a deficiency in the cystic fibrosis conductance regulator[12]. Some ATP8B1 deficiency patients also show liver steatosis and watery diarrhea. ATP8B1 deficiency can manifest with rare attacks of cholestasis or continuous cholestasis and all in between, meaning that it are not two separated diseases. There are patients who start with episodic cholestasis and progress to chronic cholestasis. Major clinical differences between patients with identical mutations suggest that there are additional factors determining the disease outcome[3]. Research on molecular function of ATP8B1 and its regulation will likely give insight into these factors.

3. ATP8B1 function

Function of ATP8B1 in ATP8B1 deficiency

The molecular function of ATP8B1 is still elusive and it remains unclear why ATP8B1 deficiency leads to intrahepatic cholestasis. In the last decade a number of possible molecular mechanisms have been postulated that link ATP8B1 to cholestasis. Here, we will describe five proposed explanations and the supporting or contraindicating evidence. First, cholestasis in ATP8B1 deficiency could be caused by defective bile salt production. However, in ATP8B1 deficiency patients, the level of bile salts in serum is high, which excludes a malfunction of bile salt synthesis[2]. Second, ATP8B1 itself could contribute directly to bile salt transport. The lack of bile salt transport in ATP8B1 transfected cells suggests that

ATP8B1 does not transport bile salts[22]. Third, impaired FXR signaling as a consequence of ATP8B1 deficiency was proposed[23]. Fourth, membrane instability due to defective ATP8B1 flippase activity was proposed to enhance cholesterol extraction resulting in decreased BSEP activity[12]. Finally, a flippase-independent effect on the expression and activity of apically targeted membrane proteins was proposed.

FXR involvement

It is suggested that FXR is downregulated due to a defect in ATP8B1. Furthermore, it is proposed that activity of signal transduction pathway components such as protein kinase C (PKC) depends on membrane asymmetry, which is mediated by ATP8B1. Loss of membrane asymmetry in ATP8B1 deficiency might affect PKC-mediated FXR modification resulting in less FXR translocation to the nucleus and less FXR activity[2, 23]. Consequently, there is an altered gene expression: repressed FXR, BSEP/ABCB11 and SHP expressions and increased expression of the ileal apical sodium-dependent bile salt transporter (ASBT). This leads to enhanced ASBT-mediated uptake of bile salts in the ileum and decreased BSEP-mediated canalicular secretion of bile salts resulting in excess bile salts in the blood and cholestasis.

Alvarez et al.[24] found a decreased FXR expression in the liver and intestine of ATP8B1 deficient patients. In one of these patients downregulation was found of genes involved in bile salt synthesis and hepatic basolateral and canalicular transport. mRNA expression of *ATP8B1* and *ABCB4* were not changed in contrast to BSEP/ABCB11 expression which had a 4-fold decrease. ABCB11 expression is strongly regulated by FXR, therefore FXR mRNA levels were measured and also SHP, a target gene of FXR. Both mRNA expression levels were found to be decreased. SHP is not expressed due to FXR downregulation. Therefore SHP cannot repress CYP7A1, leading to the changed composition of the bile salt pool seen in ATP8B1 patients: more hydrophobic CDCA synthesis and thus an increased detergent effect of the bile salt pool. These data are consistent with mRNA levels of *FXR* null mice. The similar phenotype seen in ATP8B1 deficiency patients and *FXR* null mice suggests that FXR downregulation is involved in the disease of these patients[24].

The involvement of FXR is, however, disputed for two reasons. First, FXR is also downregulated in other cholestatic diseases, together with FXR target genes discussed before. This suggests that FXR downregulation might be a secondary effect of cholestasis and not the primary mediator of cholestasis induced by ATP8B1 deficiency[25]. Second, Groen et al.[26] found no change in Fxr signaling and Asbt expression in homozygous Atp8b1 G308V mice, a model for ATP8B1 deficiency. Third, Cai et al.[8] knocked down ATP8B1 expression in human hepatocytes and found no significant change FXR expression and function. mRNA and protein expression of *FXR* or its target genes *SHP*, *ABCB11*, *Mrp2 MDR3* and *Mrp4* were not altered. They also studied FXR activity in *ATP8B1*-KO mice by adding an FXR agonist. This resulted in induced SHP, ABCB11 and MDR3 mRNA expression and decreased Mrp4 mRNA expression. These data indicate that FXR expression and function is not affected in ATP8B1 deficiency in contrast to the results in Caco-2 cells described by Chen et al. To test if the difference might lie in the fact that distinct cell types were used Cai et al performed the same study in Caco-2 cells and came to similar results as they observed in hepatocytes and mice. This indicates that changed FXR expression and function is not the primary mediator of cholestasis induced by ATP8B1 deficiency. Proinflammatory cytokines are often elevated in cholestatic diseases and can have a decreasing effect on nuclear receptor expression[27, 28]. This suggests that FXR downregulation might

be secondary to elevated proinflammatory cytokine levels and might contribute to the progression of ATP8B1 deficiency[8].

Decreased BSEP activity

It was hypothesized by Paulusma et al.[12] that in ATP8B1 deficiency an impaired aminophospholipid flippase activity leads to a loss of membrane asymmetry with more phosphatidylserine in the outer leaflet and therefore a liquid-disordered phase instead of a liquid-ordered phase. Extraction of cholesterol by hydrophobic bile salts due to the reduced membrane resistance is the consequence. This reduces the cholesterol/phosphatidylcholine (CH/PC) ratio of the outer leaflet and that might cause the decreased ABCB11 activity what eventually leads to cholestasis.

Paulusma et al.[12] showed that in Atp8b1$^{G308V/G308V}$ mutant mice the bile contained significant amounts of phosphatidylserine, an aminophospholipid that is not present in bile of control mice. This suggests that PS is situated in the outer canalicular leaflet of the hepatocyte membrane instead of the inner leaflet, where it is in healthy individuals. ATP8B1 activity could play a role in flipping PS and other aminophospholipids from the outer to the inner leaflet of the hepatocyte membrane and thereby maintaining asymmetry.

The canalicular membrane of hepatocytes of Atp8b1$^{G308V/G308V}$ mutant mice is less resistant to hydrophobic bile salts, as evidenced by PS and the elevated cholesterol and ectoenzyme in bile. After infusion of taurocholate (TC), a hydrophobic bile salt, PS is found in the mutant mice bile, but not after infusion of tauroursodeoxycholate (TUDC), a relatively hydrophilic bile salt. This suggests a role of bile salt hydrophobicity in PS extraction from the outer leaflet (see figure 1).

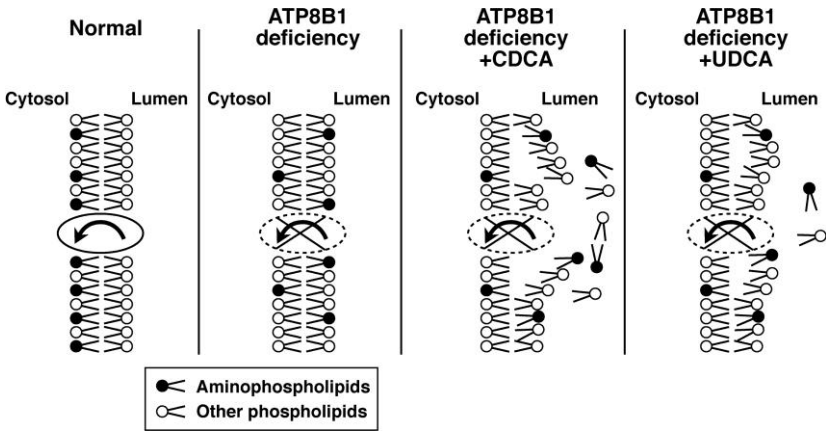


Figure 1 – ***A possible function of ATP8B1 in the hepatocytes canalicular membrane***
ATP8B1 deficiency results in a loss of aminophospholipids in the outer membrane leaflet and thus a loss of membrane asymmetry. A hydrophobic bile salt like CDCA will cause major damage to the membrane unlike a hydrophilic bile salt like UDCA[8].

The enhanced extractions are probably due to a randomized, more symmetric phospholipid distribution. The detergent effect of hydrophobic bile salts will extract more components out of the phospholipid randomized membrane. In healthy state, a membrane contains lipid rafts that are tightly packed and are therefore detergent resistant. Those lipid rafts contain a high content of sphingolipids and cholesterol and are also called a liquid-ordered phase. This asymmetry can be seen in hepatocytes, suggesting that it is then very rigid[2, 12]. In the liquid-disordered phase the membrane is less tightly packed and therefore less resistant to detergents. This phase consists of many glycerophospholipids and cholesterol and is sensitive to extraction of phospholipids, cholesterol and ectoenzymes. The symmetric membrane in ATP8B1 deficiency patients might be the result of the defect putative phosphatidylserine flippase, ATP8B1.

In other experiments Paulusma et al.[9] found that BSEP expression or localization was not changed in the mutant mice, suggesting that due to Atp8b1 deficiency not the expression but the activity of BSEP is decreased. Experiments showed that in the canalicular membrane of atp8b1-deficient ($Atp8b1^{G308V/G308V}$ mutant) mice there was a strongly decreased cholesterol/phosphatidylcholine ratio of 0,75 ± 0.24 compared to 2.03 ± 0.71 in wild-type mice, especially when challenged with taurocholate (TC). Subsequently they measured an almost linear relation between the amount of membrane cholesterol and the Abcb11 mediated ATP-dependent taurocholate transport. At the level of 80% cholesterol depletion through methyl-β-cyclodextrin (MβCD), there was no TC transport left. This could be restored by adding cholesterol. The addition of cholesterol in Atp8b1-deficient mice improved the CH/PC ratio and the ATP-dependent TC transport. These data show that the cholesterol content and CH/PC ratio in the canalicular membrane are of great importance to the Abcb11 activity and that the decrease of membrane cholesterol content in ATP8B1 deficiency might be the cause of the decreased ABCB11 activity which as a consequence causes cholestasis.

There is however no direct evidence for the PS flippase function of ATP8B1 and the exact molecular mechanism of translocation remains poorly understood. There are some questions that arise from this: 'since the secretion of bile salts from the hepatocytes to the bile duct is impaired the damaged apical membrane might not be due to toxic bile salts in the canaliculum: what does cause the apical membrane damage and PS extraction? ATP8B1 might also play a role in the membrane asymmetry of other epithelial cell membranes, because there are many extrahepatic symptoms[12].[12] ATP8B1 is essential for normal hearing[29]: 'What effect does ATP8B1 deficiency have in extrahepatic cells that do not come in contact with bile salts but nevertheless have deficiencies, such as in cochlear hair cells?' Verhulst et al.[30] investigated the flippase activity of ATP8B1 in Caco-2 cells. Blocking ATP8B1 expression with short hairpin RNA resulted in morphological changes in the apical brush border membrane but not in a decreased aminophospholipid transport or change in aminophospholipid distribution. F-actin bundles in the core of the sparse and aberrant microvilli were absent and expression of several apical membrane proteins was reduced. Reduction of protein half-life could not be measured but synthesis of apical proteins was diminished. Verhulst et al. propose that ATP8B1 affects apical protein formation and stability and that it functions as a molecular scaffold for recruiting factors necessary for microvilli formation, including actin cytoskeleton constituents.

4. Treatments

Progressive familial intraheptic cholestasis 1 and benign recurrent intrahepatic cholestasis 1 present with symptoms that start at neonatal age or infancy and eventually they will lead to liver failure and death[2, 31]. ATP8B1 deficiency thus needs to be treated to prevent death, starting at birth or infancy. There are a few medical therapies for PFIC1 patients but unfortunately the effect is limited[5-7]. However they are used in specific patients to relieve some symptoms and thereby improve quality of life and it also can prevent progression of the disease[5]. Three drugs can have a helpful effect: cholestyramine, rifampicin and ursodeoxycholic acid (UDCA). Cholestyramine is water insoluble and binds bile salts so there is no reabsoption in the enterohepatic circulation and thus less hepatic accumulation of bile salts. In patients witch episodic cholestasis this drug may shorten the episode. In PFIC1 bile salt binding resins are not very effective. Treatment with these resins only rarely relieves pruritus although sometimes the biochemical parameters are improved[5]. For rifampicin there are no

satisfactory results described in PFIC patients. However in BRIC patients it may in some patients end episodes completely[5, 18]. The third drug, UDCA, has conflicting results in ATP8B1 and ABCB11 deficiency patients. It is a hydrophilic bile salt that is less cytotoxic than endogenous bile salts and it should replace a part of the endogenous bile salts. Therefore it should reduce the damage of cholestasis to the hepatocytes membrane. The results of the UDCA treatment range from no to clear improvement[5]. However, the beneficial effect of these three drugs in BRIC patients is questionable because it is never clear if the effect is because of the drugs or because the episode spontaneously ends.

When medical treatment has no satisfying effect on clinical or biochemical parameters, more invasive treatment is necessary. There are two surgical procedures: biliary diversion (BD) and liver transplantation (LT). There are different ways to perform a BD. With Partial BD a loop of the jejunum is placed between the gall bladder and the abdominal skin (external, PEBD) or colon (internal, PIBD) to reduce accumulation of toxic bile salts by draining the bile and thus reducing the re-uptake. This results in smaller bile salt pool and a change of bile salt composition toward more hydrophilic bile salts and thus there is less injury to the canalicular membrane by hydrophobic bile salts[5, 12]. Good results have been achieved. 75% of the ATP8B1 and ABCB11 deficiency patients showed less pruritus and jaundice and a better histology. The type of mutation and the stage of liver fibrosis seem to have an influence on therapy outcome. For patients with episodic cholestasis a less invasive surgery is sufficient: a temporary nasobiliary drain (NBD) that also interrupts the enterohepatic cycle[5, 8, 32].
Some patients don't benefit from biliary diversion and will need a liver transplantation. There are high surgical risks and a lifetime of immunosuppressive drugs associated with this surgery[5]. Autoantibodies against ATP8B1 or ABCB11 might be made after LT since some patients have a mutation that leads to complete absence of the gene product. These antibodies will lead to the degradation of cells with protein ATP8B1 or ABCB11: the donated liver is rejected. This reaction has only been proved in one ABCB11 patient and not in ATP8B1 deficiency patients[2]. In ATP8B1 deficiency not only the liver is affected and therefore a liver transplantation does not resolve all the problems. In some patients with ATP8B1 deficiency diarrhea may occur or worsen after LT. A postulated explanation is that after LT there is an imbalance between bile salt secretion and absorption. In the liver the bile salts will be secreted like in healthy persons, however in the intestine ATP8B1 is still defective and might have an influence on bile salt transporters there. The resulting increased amount of bile salts in the ileum and colon causes diarrhea[5].

Because of the disadvantages of BD and LT new therapies to improve the quality of life of ATP8B1 deficiency patients are essential. In episodic and chronic ATP8B1 deficiency BD and LT are of limited use due to extrahepatic ATP8B1 expression which causes extrahepatic manifestations. Eventually these patients will suffer from malfunctioning of extrahepatic transporters. To start the search for new therapies the mutations found in patients with ATP8B1 deficiency were investigated.

5. Search for new treatment

Mutations

The *ATP8B1* gene is located at chromosome 18, locus 21[2, 3] and it encodes a ten transmembrane spanning protein (see figure 2)[1]. The gene is approximately 77 kb with 28 exons that range from 44bp

to 2.3kb each. The translation initiation codon is located in exon 2, the stopcodon in exon 28[3]. Mutations in this gene are found in both episodic and chronic intrahepatic cholestasis[2], although there is a proportion of normal GGT PFIC patients in which no mutations in ATP8B1 or ABCB11 can be found[31]. There might also be unknown mutations in introns, gene regulative regions or in genes that regulate transcription of genes involved in PFIC1 such as FXR[23, 24].

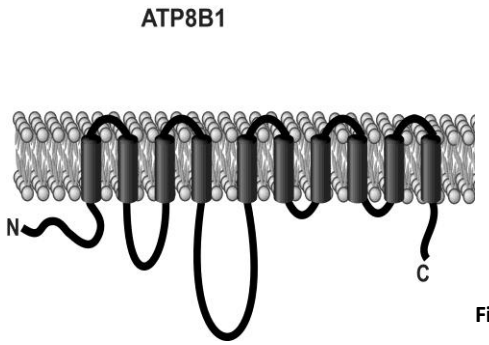


Figure 2 – ***Predicted topology of ATP8B1***

In a large study of Klomp et al.[3] the *ATP8B1* gene of BRIC and PFIC patients was sequenced and many mutations were identified that were not detectable in control samples, strongly indicating an association of these mutations with the disease. There are nonsense and missense mutations, insertions and deletions. Many patients are compound heterozygous for 2 mutations or homozygote. The type of mutation generally seems to correlate with the clinical severity of the disease. Some mutations can be found in a wide range of phenotypes from no clinical features to the most severe type of PFIC[3, 31].

PFIC patients were mainly homo- and compound heterozygous with mutations that probably affect protein expression or function severely. BRIC patients were mainly compound heterozygous with usually one mutation that only moderately affects protein expression or function, for example I661T[3]. I661T is frequent in European BRIC patients and seen both homo- and heterozygous[1, 3]. I661T is also found in PFIC patients but in combination with a different heterozygous mutation. Most missense mutations in BRIC patients are in highly conserved residues involved in ATP8B1 activity. The mutations cause a non-conserved residue substitution in functional domains such as an ATP-binding region, a hinge region, predicted transmembrane domains and the phosphorylation domain containing an aspartate residue that becomes phosphorylated for catalytic activity[3].

ATP8B1 defects

The effect of several *ATP8B1* mutations on the subcellular localization and stability of ATP8B1 and its interaction with CDC50A was recently elucidated by Folmer et al.[17] and van der Velden et al.[7] Mutations studied were PFIC1 causing missense mutations G308V, D554N and G1040R[7, 17] and BRIC1 causing mutations D70N[17] and I661T[7, 17] and the mutations D454A/G (an ATPase-dead mutant)[7, 17] and R1164X[7]. The mutations mostly affect highly conserved P4-type ATPase domains involved in protein activity or transmembrane domains[17].

Protein expression of ATP8B1 mutant proteins was often decreased while mRNA expression was unaffected[7, 17]. A clear difference could be seen in canalicular membrane expression. All PFIC1 mutants were undetectable at the canalicular membrane but showed intracellular staining presumably representing the ER, while all BRIC1 mutants had a similar staining as the wild-type. ATP8B1 I661T had despite the decreased total protein expression a similar plasma membrane

expression as wild-type ATP8B1. These data provide an explanation for the differences in severity of clinical presentation of episodic and chronic ATP8B1 deficiency[17].

Mutant protein expression levels could be restored by incubation with the proteasomal inhibitor MG-132[17]. Treatment with MG-132 or epoxomycin (also a proteasomal inhibitor) resulted in a 1.1-fold to 2-fold increase in ATP8B1 wild-type, I661T and G1040R mutants and a 2-fold to 11-fold increase in the mutants with very low protein expression: G308V, D454G, D554N an R1164X[7]. This suggests that the reduced protein levels are completely due to proteasomal degradation. This led to the hypothesis that ATP8B1 mutations result in a less stable protein and misfolded protein induced-proteasomal degradation[7, 17].

Lastly they studied the interaction of ATP8B1 mutant proteins with CDC50A by coimmunoprecipitation. CDC50A and CDC50A are proteins consisting of approximately 355 amino acids and they have 47% amino acid sequence identity, two transmembrane domains and a large exoplasmatic loop[33].

When ATP8B1 is co-expressed with CDC50A or B it is localized on the canalicular membrane instead of retained in the ER with subsequent degradation (see figure 3). Interaction of ATP8B1 with a CDC50 protein results in protein stability and increased expression. On the apical canalicular membrane ATP8B1 actively translocates NBD-PS and natural PS from the outer to the inner leaflet of the plasma membrane. ATP8B1 co-expressed with a CDC50 protein caused enhanced translocation of (NBD-)PS compared to UPS-1 cells which have no endogenous ATP8B1 or CDC50 expression. Therefore mutations interfering with the binding domains necessary for interaction of ATP8B1 with CDC50 proteins could affect plasma membrane localization and PS translocation and might cause PFIC1 or BRIC1[33].

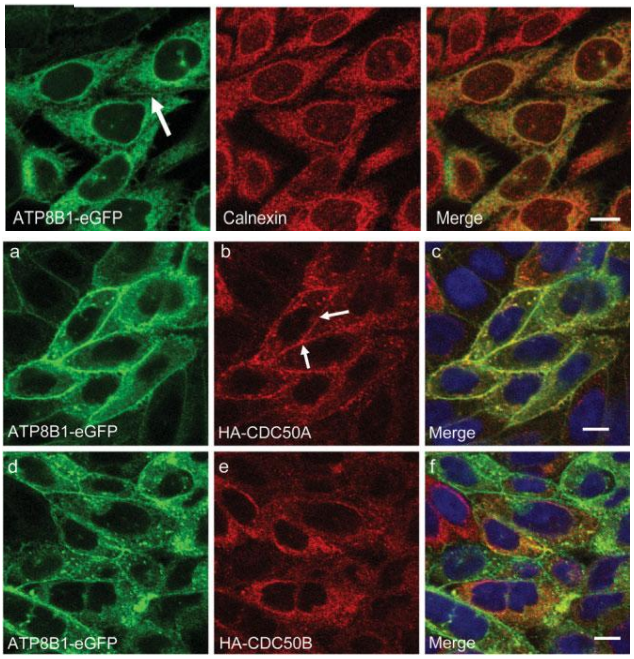


Figure 3 – ***ATP8B1-eGFP expression with or without CDC50A/B expression.***

Top: Localization of ATP8B1-eGFP with Calnexin, an ER marker, in UPS-1 cells. Weak plasma membrane ATP8B1 staining can be seen (20%, white arrow).

Middle (a-c): Localization of ATP8B1-eGFP on the plasma membrane of UPS-1 cells when co-expressed with CDC50A. Merged channel shows co-localization of ATP8B1-eGFP with CDC50A in the plasma membrane and weakly in intracellular particles.

Bottom (e-f): Localization of ATP8B1-eGFP when co-expressed with CDC50B. Staining at the plasma membrane and intracellular vesicles, respresenting late endosomes/lysosomes. Merged channel shows no clear co-localization of ATP8B1-eGFP with CDC50B.

Cells are studied with a confocal microscope. The bar represents 11.9 μm[33].

Folmer et al.[17] suggested that BRIC1 mutant proteins were coimmunoprecipitated although mutant protein interaction levels were decreased compared to wild-type, whereas most PFIC1 mutants and the ATPase-dead mutant proteins did not associate with CDC50A at all. Canalicular membrane expression was neither detectable in these mutants. Although the proteasomal inhibitor MG-132 restored protein expression, it could not restore the canalicular membrane expression of the PFIC1

mutants. These data suggest that PFIC mutations affect protein trafficking from the endoplasmatic reticulum to the plasma membrane in contrast to BRIC mutations. In contrast, Van der Velden et al.[7] did not demonstrate an affected CDC50A association due to PFIC1 or BRIC1-associated mutations. Both studies suggested that ATP8B1 mutations can result in protein misfolding and associated retention in the ER[7, 17].

Mechanism of protein folding and ER degradation

Several ATP8B1 mutations lead to a retention of the protein in the ER by the quality control system and eventually to protein degradation[17]. Thus, protein folding should be improved to increase ATP8B1 stability and subsequent plasma membrane expression[7, 17]. Accumulation of a defective misfolded protein in the cell due to retention in the ER can be toxic for the cell. Subsequent absence of the protein on its target location and failure of fulfilling its physiological function results in a disturbed cell homeostasis and threatens the viability of the patient[10, 11]. The quality control mechanism in the ER (ERQC) detects misfolded proteins and has two options to withhold them from trafficking to other compartments, including the plasma membrane: 1) returning to the protein folding machinery or 2) targeting them for degradation: ER associated protein degradation (ERAD) through the ubiquitine (UB)-proteasome pathway (UPP). Acquiring the native protein conformation does not only rely on the primary amino acid sequence: auxiliary proteins in and outside the ER, i.e. molecular chaperones are necessary. Molecular chaperones play an essential role in the quality control mechanism and help proteins to successfully pass protein folding, multidomain assembly and oligomerization in the macromolecular environment of the cell and acquire their native conformation[10].

Many diseases are related to a protein folding defect. These proteins have, however misfolded, still some residual functional activity of their wild-type counterparts if they would reach their target location instead of being recognized as aberrant by the cellular ERQC and degraded[10, 11]. This residual activity could make a major difference for the patients' viability.

Many molecular chaperones have been discovered that interact with distinct non-native protein conformations to improve protein folding. Ubiquitous 70 kDa heat shock protein (HSP70) is a major ubiquitous chaperone that binds proteins during translation to keep them in good condition for proper protein folding until the native conformation is reached and affinity is lost. Thereby it protects proteins during cellular stress and helps trafficking to other cellular compartments. Hsp70 binds the hydrophobic patches that eventually need to be located on the inside of the native protein. If the molecular chaperone fails to fold the protein properly it will target the aberrant protein to the degradation machinery. The ability to distinguish properly folded and misfolded proteins makes molecular chaperones key players of the ERQC. Hsp70 might therefore be a good therapeutic target since it interacts with many different proteins. Increased Hsp70 levels might enhance the level of products from mutated genes affecting protein folding or trafficking and improve their physiological function[10].

Heat shock transcription factor 1 (HSF1) is a transcription factor that promotes transcription of the heat shock proteins, which are almost all molecular chaperones. Besides upregulating specific molecular chaperones, increasing all molecular chaperones is also an approach to reduce protein misfolding and aggregation. Reducing the levels of specific molecular chaperones particularly involved in targeting aberrant proteins for degradation is another approach. It has been shown however that this has drastic influence on cell growth and survival and thus is not applicable[10].

In summary, there are two ways to improve protein expression: protein rescue by manipulating the ERQC compounds to escape the ERQC or protein repair by enhancing protein folding through molecular chaperones. Examples of molecular chaperones are chemical and pharmacological chaperones, also called correctors. Adding small chemical molecules to a cell can create an environment which promotes folding or prevents misfolding of mutant proteins and thus decreases progression of protein accumulation. Several small compounds are able to avoid thermally induced denaturation by stabilizing the cellular proteins, promoting proper folding and transporting the protein to its target location. Glycerol, trimethylamine N-oxide and deuterated water are such chemical chaperones. Although glycerol is effective, the concentration has to be very high and therefore it is not likely to be used clinically. Increasing expression of specific chaperone proteins might not be beneficial for the cell because pro-folding co-chaperones that cooperate with the chaperones are available in limited concentrations, the amount depending on the specific chaperone and cell type. The effect of a chaperone on a protein can therefore also differ between different cell types[10]. The pharmacological chaperones are supposed to repair aberrant enzymes or proteins by improving protein folding. Pharmacological chaperones are more specific than chemical chaperones and the interaction between the chaperone and the mutant protein is often based on the conformation of a mutant protein[10].

Most studies describing pharmacological chaperones are related to the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel. This protein is affected in patients with cystic fibrosis (CF). The ΔF508 mutation is present in the majority of these patients and results in protein misfolding and subsequent degradation which leads to the disease[34, 35].

Mutant CFTR

Cystic fibrosis (CF) is a disorder resulting in death caused by chronic lung infection and failure of lung function due to a lack of functional CFTR[34, 35]. CFTR is localized at the apical membrane of epithelial cells[34, 35] in the airways, pancreas, testis and other tissues[35]. It is activated by cAMP to regulate salt and fluid transport[34]. Internalization occurs by endocytosis whereupon it can be recycled to the plasma membrane or degraded by lysosomes. Heat shock cognate 70 (Hsc70) is a co-chaperone that can interact with the ubiquitinous chaperone Hsp70. Hsc70/Hsp70 is the first checkpoint of the ERQC for non-native CFTR and it interacts with the pro-folding Hsp40 chaperone. When folding lasts too long, Hsp40 is exchanged for a degradative factor. The second checkpoint of ERQC is ER specific calnexin. It regulates ER retention and degradation of many wild-type CFTR. Not all wild-type produced CFTR reaches the apical plasma membrane, indicating that the process is not very efficient[10].

Many mutations are found that affect the CFTR and cause cystic fibrosis. One of the known mutations, a deletion of phenylalanine at residue 508 (ΔF508) occurs in at least one allele of approximately 90% of all CF patients. The mutation affects the Nucleotide binding domain 1 (NBD1)[35]. ΔF508-CFTR is misfolded and will therefore be retained at the ER and sent to the ERAD pathway via the UPP[10, 34, 35]. In baby hamster kidney (BHK) cells it is measured that 99.5% of ΔF508-CFTR is degraded before it can reach the Golgi apparatus. However, when rescued from this degradation by incubation at low temperature or adding chemical chaperones such as glycerol, phenylbutyrate or DMSO deltaF508 CFTR has residual activity at the plasma membrane[10, 35].

Influence of temperature on protein folding

Some mutant proteins are temperature sensitive and ΔF508-CFTR is one of them. At 37˚C, the restrictive temperature, the mutant protein is rapidly degraded by ERAD and not expressed at the plasma membrane, causing the CF phenotype. The permissive temperature is 27˚C. At this temperature some of the mutant CFTR escape ERAD, are completely glycosylated and reach the plasma membrane (then called rescued, r ΔF508-CFTR) with some residual activity. When returned to 37˚C, rΔF508-CFTR is rapidly internalized and degraded. A biotinylation assay was used to measure cell surface CFTR. Biotinylated CFTR was found in 27˚C culture cells but not in 37˚C cultured cells. In polarized epithelia, wild-type CFTR was stable unlike rΔF508-CFTR. rΔF508-CFTR half-life was 3-fold shorter than wild-type CFTR (4 ± 1h and 12 ± 1,5h respectively), the cAMP response and channel activity were decreased and internalization rate was increased. This however could be avoided by culturing at 27˚C. When kept at this temperature, the half-lifes of wild-type and ΔF508-CFTR were equal, cAMP respons was restored and internalization rate reduced[36]. Van der Velden et al.[7] also showed that low temperature incubation increases expression of ATP8B1 mutant protein.

Pharmacological chaperones

The stability of low temperature rescued ΔF508-CFTR (rΔF508-CFTR) is still impaired. Pharmacological chaperones, from now called correctors, could help improving mutant protein function by promoting protein folding and subsequent trafficking to the site of action[35].

Many studies are conducted to find correctors that either stabilize the tertiary structure of ΔF508-CFTR or alter the binding to specific ER chaperones to prevent ER retention and subsequent degradation. Several correctors have been found to have a beneficial effect on ΔF508-CFTR function, including 4-phenylbutyrate (4-PBA), corr-1a, 2a, 3a, 4a and -2b, CFcorr-325 and benzoquinolizinium (MPB) derivatives[11, 34, 35]. Identification and characterization of these molecules is often achieved by high-throughput screening.

Treatment with 4-PBA, a clinically approved corrector, leads to decreased Hsc70 and consequent improved trafficking of ΔF508-CFTR[11]. MPB derivatives are correctors specific for ΔF508-CFTR and correct the trafficking defect by binding the protein and preventing proteasomal degradation[11]. CFcorr-325 and corrector 2b promote protein folding of ΔF508-CFTR. CFcorr-325 promotes interaction between transmembrane domains TMD1 and 2 plus insertion of a transmembrane part in the membrane in the correct orientation. CFcorr-325 thereby facilitates protein folding to its native conformation. Corrector 4a is a bisaminomethylbithiazole compound that also enhances proper folding of ΔF508-CFTR[34].

High-throughput screening by Pedemonte et al.[35] yielded four correctors. Several of these correctors improved ΔF508-CFTR folding, stability, plasma membrane expression and channel function measured by looking at the chloride current. These changes were greater than seen in 27˚C-incubated or 4-PBA treated cells. Corrector 4a showed the best improvements. Corrector-treated cells showed a significantly higher chloride current, some correctors caused a 3-fold increase in ΔF508-CFTR folding efficiency and 5-fold to 6-fold increases in plasma membrane expression. In epithelial cells expressing ΔF508-CFTR the onset of the correction after corrector addition was faster and lasted longer than in the 27˚C incubated or 4-PBA treated cells (see figure 4, right)[35].

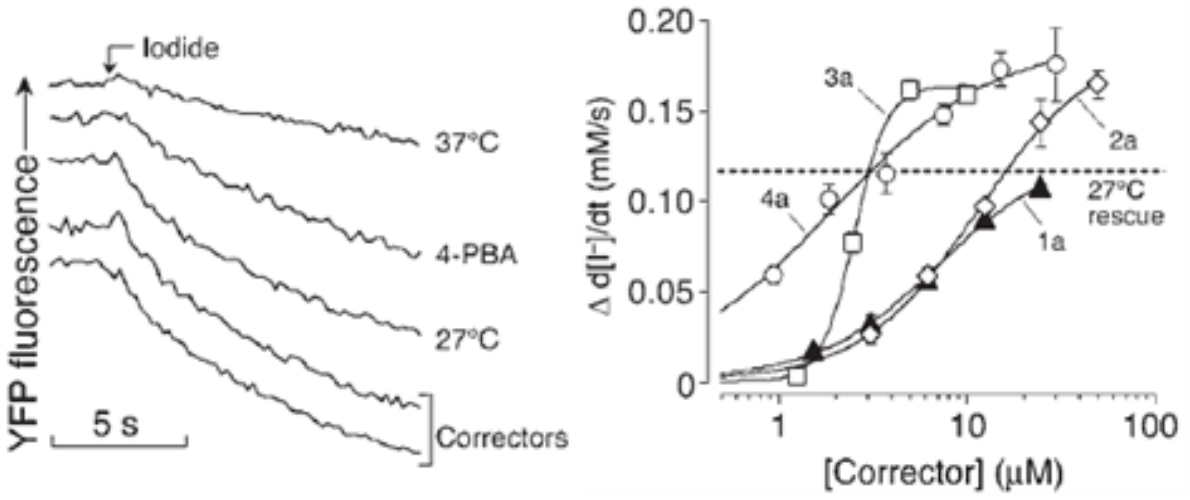


Figure 4 – *Iodide influx in ΔF508-CFTR FRT cells*
Cells were co-expressed with ΔF508-CFTR and a halide-sensitive yellow fluorescent protein (YFP). Left: iodide influx at different conditions. Right: dose-dependent response of different correctors. Dashed line is the positive control: low-temperature rescued ΔF508-CFTR activity[35].

In contrast to wild-type CFTR, ΔF508-CFTR fails to become complex glycosylated under untreated conditions. When treated with correctors for several hours at 37˚C, an accumulation of complex-glycosylated ΔF508-CFTR can be seen in ΔF508-CFTR expressing cells. Furthermore, results from experiments by Varga et al.[36] conducted in polarized airway epithelial cells indicated that corr-4a and also CFcorr-325 decreased the internalization rate in rΔF508-CFTR from 30% to approximately 1% and 5% respectively although they had no effect on wild-type CFTR internalization. When returning to 37˚C after low temperature rescued ΔF508-CFTR, the plasma membrane abundance decreased to 35% after 3 hours, in contrast to 50-60% when correctors 3a, 4a or 4b were added[35]. Corr-2a is the best stabilizer although it has no effect on protein folding; corrector 4 has both abilities. In human homozygous ΔF508-CFTR bronchial epithelial cells only corr-4a resulted in a faintly increased chloride current, the other correctors showed no significant increase[35].
Regarding these data it is suggested that correctors assist in protein folding and increase mature ΔF508-CFTR stability. They do not enhance protein synthesis but rather proper folding and trafficking to the plasma membrane. It is seen that the class 4 correctors cause a 2-fold to 3-fold increase in ΔF508-CFTR folding efficiency and a 30-50% decrease in ER degradation[35]. In this study several correctors appeared useful for protein folding. Some of these could possibly be helpful in other diseases that are caused by protein misfolding.

EXPERIMENTS

1. Introduction

4-PBA and ATP8B1 expression

Some CFTR correctors might also improve ATP8B1 folding which could be beneficial to treat ATP8B1 patients. To test this possibility van der Velden et al.[7] studied the effect of 4-phenylbutyrate (4-PBA) on ATP8B1 plasma membrane expression. 4-PBA is a clinical approved pharmacological chaperone that has become a promising drug to improve plasma membrane expression of misfolded proteins in genetic disorders originating or manifesting in the liver. Seven ATP8B1 mutations were selected to test the influence of 4-PBA in human bone osteosarcoma epithelial cells (U2OS). In ATP8B1 G308V transgenic cells 4-PBA dose-dependent significant increases of ATP8B1 expression were found (see figure 5, left (A)).

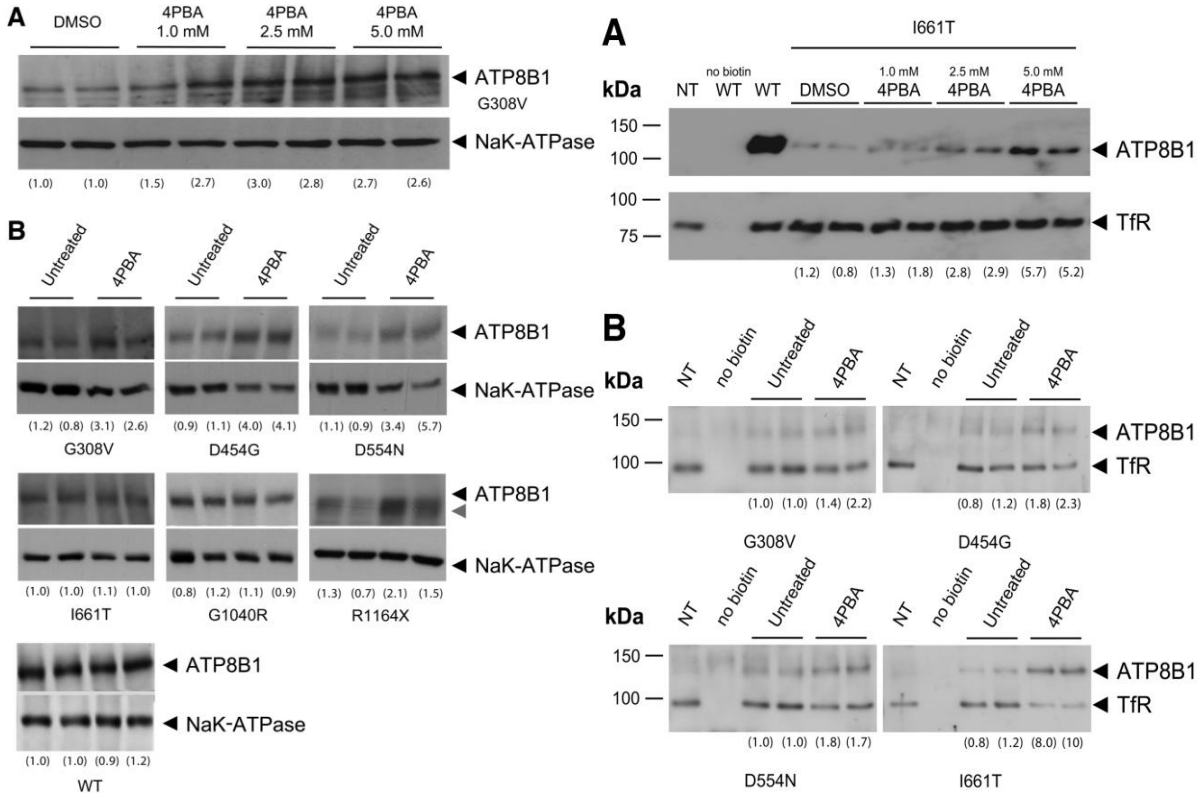


Figure 5 – *ATP8B1 expression after 4-PBA treatment*

Cells were cotransfected with ATP8B1 and CDC50A cDNA and incubated witch 4-PBA for 40h.

Left: Total ATP8B1 expression was measured in cell lysates by Western blot and densitometry (ImageJ). Signal intensity was corrected for protein loading (NaK-ATPase). Below each panel the average expression compared to control condition is shown. A: ATP8B1 G308V, treated with different amounts of 4-PBA. B: Various mutants treated with 4-PBA.

Right: ATP8B1 plasma membrane expression was measured with a biotinylation assay and subsequent a western blot and densitometry (ImageJ). Signal intensity was corrected for protein loading (TfR). Below each panel the average expression compared to control condition is shown. A: ATP8B1 I661T, treated with different amounts of 4-PBA. B: Various mutants treated with 4-PBA[7].

Total cellular ATP8B1 expression in 4 of the 7 transgenic cell types was increased 2-fold to 5-fold after 4-PBA treatment: G308V, D454G, D554N and R1164X (see figure 5 left (B)). Of these, the first three also showed a 1.5-fold to 2-fold increase in plasma membrane expression (see figure 5 right (B)) while ATP8B1 R1164X was not detectable at the plasma membrane both before and after 4-PBA treatment. Protein levels of I661T and G1040R ATP8B1 were only faintly reduced and 4-PBA treatment did not cause much increase. However, the abundance of ATP8B1 I661T at the plasma membrane was 5-fold to 10-fold increased upon 4-PBA treatment. Wild-type ATP8B1 was not affected at all by 4-PBA.

The results suggest that enhanced plasma membrane expression as a consequence of treatment with pharmacological chaperones might be mutation specific. Further research should be done with different correctors to find the best corrector for each mutation found in ATP8B1. BRIC1 patients only have episodic attacks and in the meantime there are no symptoms, suggesting that partial recovery of ATP8B1 plasma membrane expression could be sufficient for clinical improvement.

Corrector administration might be a promising treatment to relieve the symptoms of ATP8B1 deficiency, especially in the episodic form. We therefore tested the effect of three correctors C1, 12 and 16 on the expression of one common BRIC1 mutation, ATP8B1 I661T.

2. Materials and methods

Cell lines, cell culture and transfection.

Human bone osteosarcoma epithelial cells (U2OS), which do not endogenously express ATP8B1, were cultured in Dulbeco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin in a 37˚C humidified incubator in 5% CO_2. For immunocytochemistry cells were grown on glass coverslips in a 24-well cell culture plate and transiently transfected with (150 ng/well) HA-ATP8B1 or HA-ATP8B1 (wild-type or I661T mutation) and (7,5ng/well) CDC50A-V5 using pcDNA3 and PEI. For the corrector treatment experiment transient transfections with 4µg/10cm Petri Dish HA-ATP8B1 I661T or 4µg/10cm Petri Dish HA-ATP8B1 I661T and 200ng/10cm Petri Dish CDC-50A-V5 were performed using pcDNA3 and PEI. Cells were incubated at 37˚C for 48 hours.

Immunocytochemistry.

Cells were fixed in 4% paraformaldehyde (PFA) in pH 7.4 phosphate buffered saline (PBS) for 20 minutes at room temperature (RT). Cells were permeabilized with 0.2% Triton X-100 in PBS and quenched with NH_4Cl and blocked with 2%BSA. Then cells were incubated with mouse-anti-HA antibodies (1:2000) (Sigma, St.Louise, MO) in 2%BSA. The immunoreactivity was visualized with goat-anti-mouse Alexa-568 (1:300) (Molecular Probes, Breda, Netherlands) in 2%BSA and mouse-anti-FITC-V5 (1:500) (Sigma) in 2%BSA. Nuclei were stained with (1mg/ml) DAPI and samples were mounted in mowiol. Images were obtained using Zeiss LSM confocal microscope.

Molecular correctors (pharmacological chaperones).

Correctors were provided by the CFTR Folding Consortium (http://www.cftrfolding.org). Correctors tested were C1 (6-(1H-Benzoimidazol-2-ylsulfanylmethyl)-2-(6-methoxy-4-methyl-quinazolin-2-ylamino)-pyrimidin-4-ol), C12 (2i, aminoarylthiazole, N-(4-fluorophenyl)-4-p-tolylthiazol-2-amine) and C16 (3d, quinazolinylaminopyrimidinone, 2-(6-methoxy-4-methylquinazolin-2-ylamino)-5,6-

dimethylpyrimidin-4(1H)-one). C1, C12 and C16 were used at 6µM, 10µM and 10µM in 0.1% DMSO/DMEM respectively. Cells were incubated with one corrector for 40 hours. In all experiments, control samples were incubated with 0.1% DMSO.

Cell surface biotinylation.

Two days after incubation with correctors U2OS cells were washed with PBS supplemented with 0.4mM $CaCl_2$ and 1.0 mM $MgCl_2$ (PBS-CM) and protease inhibitor. Cell-surface proteins were biotinylated by incubation with 0.5 mg/mL sulfo-NHS-SS-biotin for 30 minutes at 4˚C and then washed with PBS-CM supplemented with 0.1% BSA. Cells were lysed in 20mM Tris-HCl; pH7.4, 5mM NaEDTA, 135mM NaCl, 1.0% (v/v) Nonidet P-40, and 10% (w/v) sucrose and scraped and centrifuged at 14.000rpm for 10m at 4˚C. 70µL of each sample was used for Western Blotting by adding sample buffer (SB, with 33% DTT). Protein concentration in cell lysate was measured with a BCA protein assay kit. Biotinylated proteins were precipitated for 2h at RT using NeutrAvidin-coupled beads (Pierce). Precipitated proteins were eluted with SB and analyzed by immunoblot analysis.

Western blot analysis.

To quantify total and cell surface expressed HA-ATP8B1 unprecipitated and precipitated protein samples were analyzed by SDS/PAGE (6% gels) and Western blotting. PVDF membranes were blocked for one hour in 2,5% milk/TBS-T at RT. HA-ATP8B1 was detected with α-HA-HRP (1:1000) antibody (Sigma) overnight at 4˚C in 2,5% milk/TBS-T. Protein loading was checked with mouse-α-Transferrine (1:2000, Zymed) for 2h at RT and secondary goat-α-mouse-HRP antibody (1:1000) (Dako, Enschede, Netherlands) in 2,5% milk for 1h at RT. Total and plasma membrane expressed HA-ATP8B1 was detected with HRP substrate enhanced chemiluminescence (ECL, 1ml/membrane). The Western blots were analyzed and densities measured using ImageJ.

3. Results

ATP8B1 needs CDC50A to reach the plasma membrane.

Endogenously expressed wild-type ATP8B1 traffics to the apical plasma membrane in epithelial cells and hepatocytes. With immunocytochemistry we tested ATP8B1 trafficking in U2OS cells when expressed individually with HA-ATP8B1 and found it to be exclusively localized intracellular, representing the ER (see figure 6). However, when co-expressed with its interaction partner CDC50A-V5, HA-ATP8B1 traffics to the plasma membrane and localizes there, partially with CDC50A-V5 (see figure 7). This suggests that CDC50A is necessary for ATP8B1 to reach the cell surface.

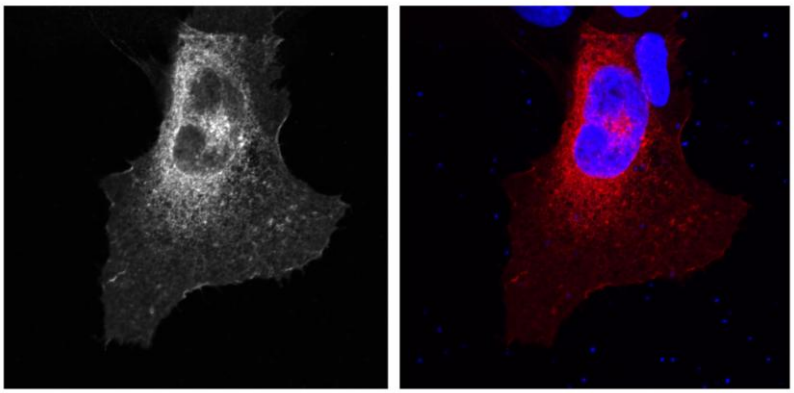


Figure 6 – *ATP8B1 mislocalization without CDC50A co-expression*
Cells were transfected with solely HA-ATP8B1 and visualized with primary mouse-α-HA and secondary goat-anti-mouse Alexa-568(red). Nucleus is stained with DAPI (blue). ATP8B1 is detected in intracellular particles, representing the ER. Left: ATP8B1 expression, right: merged channel.

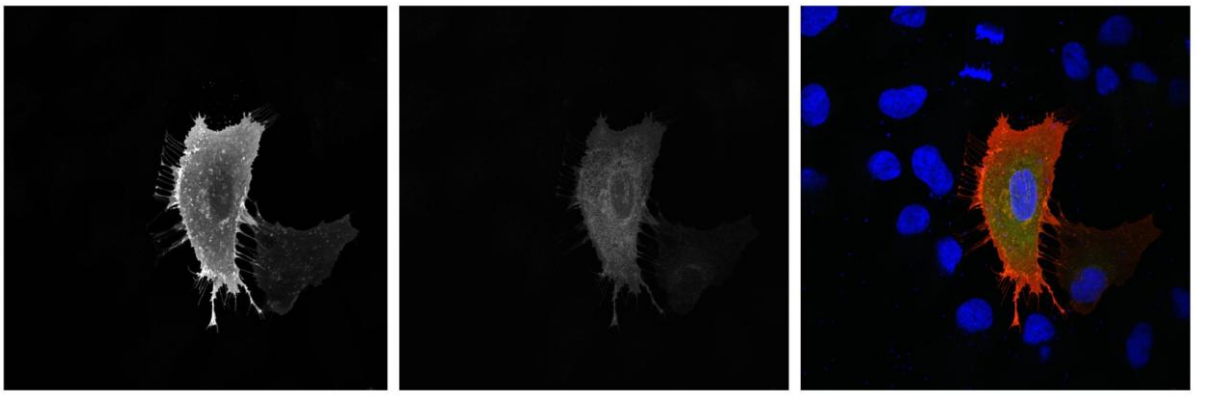


Figure 7 – *Plasma membrane localization of ATP8B1 when co-expressed with CDC50A*
Cells were cotransfected with HA-ATP8B1 and CDC50A-V5. HA-ATP8B1 was visualized with primary mouse-α-HA and secondary goat-anti-mouse Alexa-568(left, red). CDC50A-V5 was visualized with secondary mouse-anti-FITC-V5 (middle, green). Nucleus is stained with DAPI (blue). ATP8B1 is detected at the plasma membrane, partially co-localized with CDC50A. CDC50A is not specifically detected at the plasma membrane. Left: ATP8B1, middle: CDC50A, right: merged channels.

Mislocalization of ATP8B1 I661T

To test protein trafficking of the ATP8B1 I661T, its expression pattern was compared with wild-type ATP8B1 in U2OS cells by immunocytochemistry. Cells were co-transfected with HA-ATP8B1 I661T and CDC50A-V5. In contrast to wild-type ATP8B1 the ATP8B1 I661T protein was not detectable at the plasma membrane (see figure 8). Intracellular ATP8B1 staining was seen completely co-localized with CDC50A, probably representing the ER.

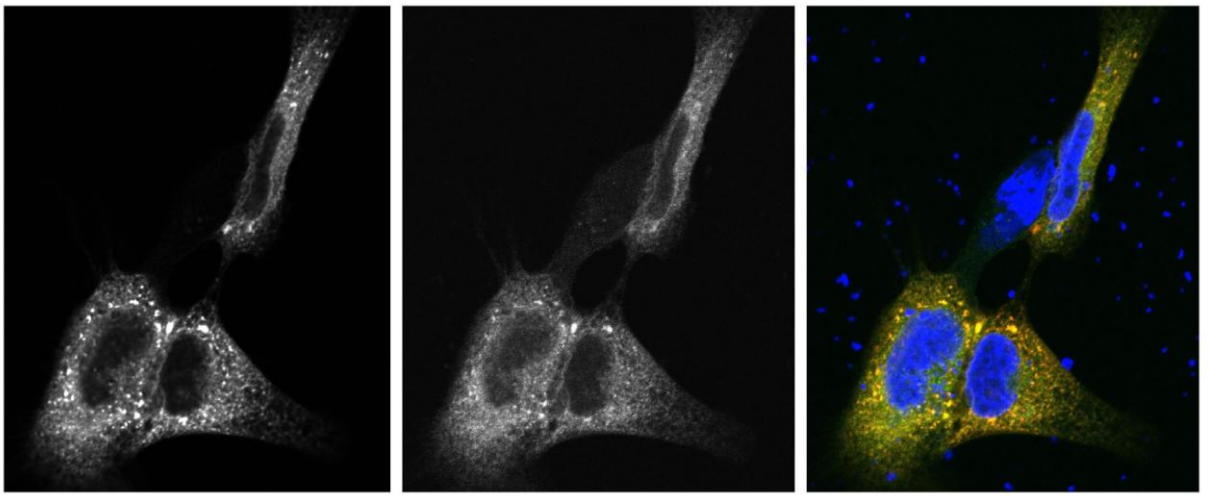


Figure 8 – *Localization of mutant ATP8B1 in transiently transfected U2OS cells by immunocytochemistry*
HA-ATP8B1 I661T and CDC50A-V5 were cotransfected in U2OS cells. HA-ATP8B1 was visualized with primary mouse-α-HA and secondary goat-anti-mouse Alexa-568(red). CDC50A-V5 was visualized with mouse-anti-FITX-V5 (green). Nucleus stained with DAPI (blue). ATP8B1 is detected at intracellular particles, co-localized with CDC50A. Left: ATP8B1, middle: CDC50A, right: merged channels.

Corrector-improved plasma membrane localization of ATP8B1 I661T

To investigate the role of 40 hours incubation with one of the three correctors C1, C12 and C16 on plasma membrane expression of ATP8B1 I661T, total and plasma membrane expression in U2OS cells was measured with cell surface biotinylation. After biotinylation we detected biotinylated ATP8B1 I661T by Western blot analysis. This way only ATP8B1 I661T expressed at the plasma membrane and not intracellular, misfolded ATP8B1 I661T will be detected, unless the membrane is permeabilized. In the unprecipitated fraction the difference between not biotinylated (NB) and biotinylated ATP8B1 I661T (I661T) indicates that biotinylation affects HA-ATP8B1 I661T expression. The NeutrAvidin-coupled beads specifically bind biotinylated proteins, demonstrated by the absence of the plasma membrane transferrin receptor (NB, TfR) staining and HA-ATP8B1 staining (NB, HA-ATP8B1) in the not biotinylated (NB) precipitated fraction (see figure 9).
Corrector C16 caused much cell death (not shown) and when looking at the western blot (see figure 9), it looked like the corrector had a decreasing effect on ATP8B1 I661T plasma membrane expression. However, when corrected for protein loading by measuring transferrin receptor expression, we observed that C16 enhanced total ATP8B1 I661T expression by 28% (±16%) and plasma membrane expression with 78% (±47%) (See figure 10). C12 showed little cell death (not shown) and not much change in both total and plasma membrane expression could be seen (see figure 9). Also when looking at the signal intensities no change in total ATP8B1 I661T expression could be seen after C12 incubation (see figure 10) and even a 37% (±16%) decrease in plasma membrane expression, making this drug not suitable. C1 also showed some cell death (not shown) but an increase in total and plasma membrane ATP8B1 I661T expression can be seen (see figure 9). A 31% (±14%) increase in total and even 91% (±7%) in plasma membrane expression can be seen (see figure 10), making C1 a potential promising drug for this disease.

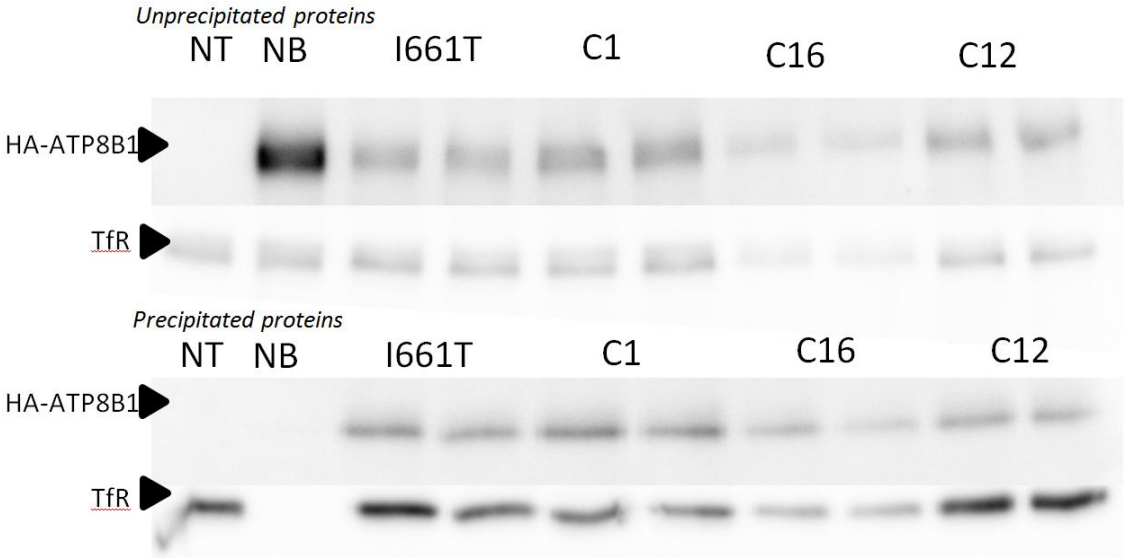


Figure 9 – *Corrector effect on total and plasma membrane expression of ATP8B1 I661T*
Cells were co-transfected with HA-ATP8B1 I661T and CDC50A and treated with 0.1% DMSO (solvent) or corrector C1, C16 or C12 for 40h at 37˚C. Subsequently a biotinylation was conducted followed by Western blotting and coimmunoprecipitation with α-HA-HRP plus ECL (upper two panels) to display the effect of the correctors on total mutant ATP8B1 I661T expression. In the bottom two panels only cell surface biotinylated proteins, precipitated with NeutrAvidin-coupled beats, were Western blotted and coimmunoprecipitated to investigate the effect of the correctors on plasma membrane expression of ATP8B1 I661T. Protein loading was verified using transferrin receptor (TfR) expression, measured by primary mouse-α-TfR and secondary goat-α-mouse-HRP antibody plus ECL..

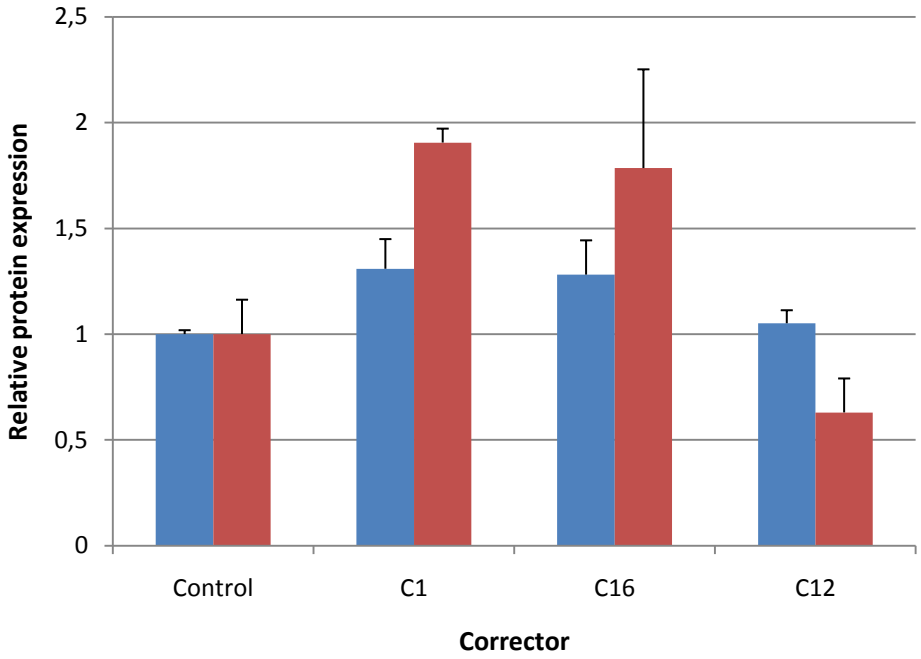


Figure 10 – *Corrector effect on mutant HA-ATP8B1 I661T plasma membrane expression*.
To facilitate comparison of protein expression seen in figure 10, ATP81 signal intensity of the experiment shown was quantified using ImageJ. Signal intensity was corrected for protein loading using the Transferrin receptor signal and for background signal and presented relative to the average of the control non-treated ATP8B1 I661T cells. Experiments were performed in duplicates. The results are expressed as means ± the standard deviation (n=2) Blue: cell lysate expression, red: cell surface expression.

4. Discussion

Mutations in ATP8B1 result in benign recurrent intrahepatic cholestasis type 1 (BRIC1) or progressive familial intrahepatic cholestasis type 1 (PFIC1). Because ATP8B1 is not only expressed in the liver but also in other tissues[8] common treatment is not sufficient. Current drugs are not successful for every patient and biliary diversion and liver transplantation do not ameliorate extrahepatic symptoms[5].
Therefore we searched for other drugs. Since mutations in *ATP8B1* often lead to protein misfolding and subsequent retention in the ER and degradation of the protein[7] mutated ATP8B1 does not reach the plasma membrane while it often still has some residual activity[10, 11]. First we studied the interaction between ATP8B1 and CDC50A. ATP8B1 needs CDC50A to exit the ER and travel to the plasma membrane. However, ATP8B1 I661T does not reach the plasma membrane despite the co-localization with CDC50A.(This study and [7, 17, 33]) They are co-localized in the ER, indicating two explanations. First, the binding site is not mutated in ATP8B1 I661T and interaction with CDC50A is so strong that it is pulled into the degradative pathway. Second, unstable ATP8B1 I661T also makes CDC50A unstable which causes it to be degraded. This could be investigated by performing a coimmunoprecipitation with mutant ATP8B1 and CDC50A to check the interaction between these two proteins.
After this we studied the impact of three pharmacological chaperones on total and plasma membrane expression of the common BRIC1 mutation ATP8B1 I661T since these chaperones are known to improve cystic fibrosis conductance regulator (CFTR) folding, stability and trafficking[11, 34, 35]. C12 had no or a reducing effect on total and plasma membrane expression of ATP8B1 I661T while C1 and C16 had an increasing effect on both total and plasma membrane expression. This suggests that C1 and C16 enhance protein folding and thereby also the trafficking to the plasma membrane. Nonetheless corrector C16 is not suitable for clinical use since it is so toxic for the cells. C1 improved total ATP8B1 I661T only by 31% while plasma membrane expression increased by 91%, suggesting that this corrector particularly enhances trafficking of the mutant protein to the site of action. Since corrector C1 caused the lowest cell death and increased the total and cell surface expression of this mutant protein it might be the most promising drug for treating patients with ATP8B1 deficiency with an I661T mutation and relieving their symptoms. Especially BRIC1 patients might benefit from this corrector because the I661T mutation occurs in many European patients with this type of intrahepatic cholestasis.
C16 caused much cell death and the cells had a different morphology. As a result of the cell death, the total ATP8B1 I661T amount left was only 180µg. Therefore the amount of protein loaded for the western blot had to be very low for all the corrector-treated cells. The results are thus not optimal. To improve the quality of the results, we could repeat the experiments without corrector C16.
In addition considerations of future investigations include: First, future research should optimize the C1 dosing concentrations to reduce the toxic effect on cells. This can be done by adding different C1 concentrations to cells with the same mutation. Second, the functional activity of corrector rescued ATP8B1 I661T should be tested to see if increased ATP8B1 I661T plasma membrane expression that is better folded will make difference, also for BSEP activity, since this is decreased in ATP8B1 deficiency hepatocyte cells. Rescued mutant ATP8B1 activity might be investigated by looking at aminophospholipid flipping or the membrane composition. BSEP activity can be tested by looking at bile salt transport to the apical outside of the cell. Third, the effect of correctors on human patient hepatocytes and other human cells expressing ATP8B1 should also be tested, for example intestinal, pancreatic and cochlear cells, because we are looking for a treatment that also reduces extrahepatic

symptoms. Fourth, the effect of correctors on whole tissues by experimenting in tissue models and animal models has to be tested. This will elucidate if the bile salt homeostasis improves and symptoms are relieved. Fifth, 4-PBA had the best effect on the I661T mutation[7], suggesting that there also might be a specific corrector for each ATP8B1 mutation that increases its plasma membrane expression the most. The effect of C1 and other correctors on distinct ATP8B1 mutations should be investigated to find a specific corrector treatment for each ATP8B1 mutation and thereby optimize the treatment for each individual patient with ATP8B1 deficiency. Combining 4-PBA and C1 might also further improve the beneficial effect. Using multiple specific correctors for two distinct mutations of one patient might make the treatment optimal.

ACKNOWLEDGEMENTS

I thank my supervisor during this research project: Dr. Stan van de Graaf, for directing and supporting me during this process. Also I thank my tutors during the experiments: Ingrid Bijsmans, Phd and Wendy van der Woerd, MD. Lastly, I thank my second supervisor Dr. Joost Koedam, for directing me the last week.

REFERENCES

1. Bull, L. N. *et al*. A gene encoding a P-type ATPase mutated in two forms of hereditary cholestasis. *Nat. Genet.* **18**, 219-224 (1998).

2. Hori, T., Nguyen, J. H. & Uemoto, S. Progressive familial intrahepatic cholestasis. *Hepatobiliary. Pancreat. Dis. Int.* **9**, 570-578 (2010).

3. Klomp, L. W. *et al*. Characterization of mutations in ATP8B1 associated with hereditary cholestasis. *Hepatology* **40**, 27-38 (2004).

4. Bull, L. N. *et al*. Fine-resolution mapping by haplotype evaluation: the examples of PFIC1 and BRIC. *Hum. Genet.* **104**, 241-248 (1999).

5. van der Woerd, W. L. *et al*. Familial cholestasis: progressive familial intrahepatic cholestasis, benign recurrent intrahepatic cholestasis and intrahepatic cholestasis of pregnancy. *Best Pract. Res. Clin. Gastroenterol.* **24**, 541-553 (2010).

6. Tygstrup, N., Steig, B. A., Juijn, J. A., Bull, L. N. & Houwen, R. H. Recurrent familial intrahepatic cholestasis in the Faeroe Islands. Phenotypic heterogeneity but genetic homogeneity. *Hepatology* **29**, 506-508 (1999).

7. van der Velden, L. M. *et al*. Folding defects in P-type ATP 8B1 associated with hereditary cholestasis are ameliorated by 4-phenylbutyrate. *Hepatology* **51**, 286-296 (2010).

8. Cai, S. Y. *et al*. ATP8B1 deficiency disrupts the bile canalicular membrane bilayer structure in hepatocytes, but FXR expression and activity are maintained. *Gastroenterology* **136**, 1060-1069 (2009).

9. Paulusma, C. C., de Waart, D. R., Kunne, C., Mok, K. S. & Elferink, R. P. Activity of the bile salt export pump (ABCB11) is critically dependent on canalicular membrane cholesterol content. *J. Biol. Chem.* **284**, 9947-9954 (2009).

10. Amaral, M. D. Therapy through chaperones: sense or antisense? Cystic fibrosis as a model disease. *J. Inherit. Metab. Dis.* **29**, 477-487 (2006).

11. Norez, C., Bilan, F., Kitzis, A., Mettey, Y. & Becq, F. Proteasome-dependent pharmacological rescue of cystic fibrosis transmembrane conductance regulator revealed by mutation of glycine 622. *J. Pharmacol. Exp. Ther.* **325**, 89-99 (2008).

12. Paulusma, C. C. *et al*. Atp8b1 deficiency in mice reduces resistance of the canalicular membrane to hydrophobic bile salts and impairs bile salt transport. *Hepatology* **44**, 195-204 (2006).

13. Stedman, C. *et al*. Benefit of farnesoid X receptor inhibition in obstructive cholestasis. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 11323-11328 (2006).

14. Chawla, A., Saez, E. & Evans, R. M. "Don't know much bile-ology". *Cell* **103**, 1-4 (2000).

15. Wang, H., Chen, J., Hollister, K., Sowers, L. C. & Forman, B. M. Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. *Mol. Cell* **3**, 543-553 (1999).

16. Crawley, M. L. Farnesoid X receptor modulators: a patent review. *Expert Opin. Ther. Pat.* **20**, 1047-1057 (2010).

17. Folmer, D. E., van der Mark, V. A., Ho-Mok, K. S., Oude Elferink, R. P. & Paulusma, C. C. Differential effects of progressive familial intrahepatic cholestasis type 1 and benign recurrent intrahepatic cholestasis type 1 mutations on canalicular localization of ATP8B1. *Hepatology* **50**, 1597-1605 (2009).

18. van Ooteghem, N. A., Klomp, L. W., van Berge-Henegouwen, G. P. & Houwen, R. H. Benign recurrent intrahepatic cholestasis progressing to progressive familial intrahepatic cholestasis: low GGT cholestasis is a clinical continuum. *J. Hepatol.* **36**, 439-443 (2002).

19. Strautnieks, S. S. *et al*. A gene encoding a liver-specific ABC transporter is mutated in progressive familial intrahepatic cholestasis. *Nat. Genet.* **20**, 233-238 (1998).

20. van der Velden, L. M., van de Graaf, S. F. & Klomp, L. W. Biochemical and cellular functions of P4 ATPases. *Biochem. J.* **431**, 1-11 (2010).

21. Dawson, P. A. Liver disease without flipping: new functions of ATP8B1, the protein affected in familial intrahepatic cholestasis type 1. *Hepatology* **51**, 1885-1887 (2010).

22. Harris, M. J., Kagawa, T., Dawson, P. A. & Arias, I. M. Taurocholate transport by hepatic and intestinal bile acid transporters is independent of FIC1 overexpression in Madin-Darby canine kidney cells. *J. Gastroenterol. Hepatol.* **19**, 819-825 (2004).

23. Chen, F. *et al*. Progressive familial intrahepatic cholestasis, type 1, is associated with decreased farnesoid X receptor activity. *Gastroenterology* **126**, 756-764 (2004).

24. Alvarez, L. *et al*. Reduced hepatic expression of farnesoid X receptor in hereditary cholestasis associated to mutation in ATP8B1. *Hum. Mol. Genet.* **13**, 2451-2460 (2004).

25. Demeilliers, C. *et al*. Altered hepatobiliary gene expressions in PFIC1: ATP8B1 gene defect is associated with CFTR downregulation. *Hepatology* **43**, 1125-1134 (2006).

26. Groen, A. *et al*. Intestinal bile salt absorption in Atp8b1 deficient mice. *J. Hepatol.* **47**, 114-122 (2007).

27. Plebani, M. *et al*. Cytokines and the progression of liver damage in experimental bile duct ligation. *Clin. Exp. Pharmacol. Physiol.* **26**, 358-363 (1999).

28. Zachou, K. *et al*. Autoimmune hepatitis type 1 and primary biliary cirrhosis have distinct bone marrow cytokine production. *J. Autoimmun.* **25**, 283-288 (2005).

29. Stapelbroek, J. M. *et al*. ATP8B1 is essential for maintaining normal hearing. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 9709-9714 (2009).

30. Verhulst, P. M. *et al*. A flippase-independent function of ATP8B1, the protein affected in familial intrahepatic cholestasis type 1, is required for apical protein expression and microvillus formation in polarized epithelial cells. *Hepatology* **51**, 2049-2060 (2010).

31. Davit-Spraul, A. *et al*. ATP8B1 and ABCB11 analysis in 62 children with normal gamma-glutamyl transferase progressive familial intrahepatic cholestasis (PFIC): phenotypic differences between PFIC1 and PFIC2 and natural history. *Hepatology* **51**, 1645-1655 (2010).

32. Stapelbroek, J. M. *et al*. Nasobiliary drainage induces long-lasting remission in benign recurrent intrahepatic cholestasis. *Hepatology* **43**, 51-53 (2006).

33. Paulusma, C. C. *et al*. ATP8B1 requires an accessory protein for endoplasmic reticulum exit and plasma membrane lipid flippase activity. *Hepatology* **47**, 268-278 (2008).

34. Wang, Y., Loo, T. W., Bartlett, M. C. & Clarke, D. M. Modulating the folding of P-glycoprotein and cystic fibrosis transmembrane conductance regulator truncation mutants with pharmacological chaperones. *Mol. Pharmacol.* **71**, 751-758 (2007).

35. Pedemonte, N. *et al*. Small-molecule correctors of defective DeltaF508-CFTR cellular processing identified by high-throughput screening. *J. Clin. Invest.* **115**, 2564-2571 (2005).

36. Varga, K. *et al*. Enhanced cell-surface stability of rescued DeltaF508 cystic fibrosis transmembrane conductance regulator (CFTR) by pharmacological chaperones. *Biochem. J.* **410**, 555-564 (2008).