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The role of ABCC1 in modulating drug sensitivity in acute myeloid leukemia (AML)

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submitted by

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1. Introduction

1.1. Leukemia

Leukemia is a malignant disease of the hematopoietic system and is characterized by the expansion of abnormal white blood cells. Depending on the time span of disease progression and the maturity of the leukemic cells, leukemias can be divided into acute and chronic forms. Moreover, the cell type of origin allows for distinction between lymphoblastic and myelogenous leukemias. This leads to four major types of leukemia: acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CML) and chronic myeloid leukemia (CML). Leukemias are a rare type of malignancy, representing around 4% of all newly diagnosed cancer types each year (Siegel et al. 2019). Nevertheless, acute leukemias account for over 20% of pediatric cancer cases – 80% of which are ALL cases and 20% are AML cases – hence they are one of the most common cancer entities in children (Puumala et al. 2013). In general, acute leukemias have a worse prognosis than chronic leukemias.

1.2. Acute Myeloid Leukemia (AML)

AML is an aggressive form of leukemia that is characterized by increased numbers of immature myeloid progenitor cells, so called myeloblasts. The accumulation of myeloblasts in the bone marrow and blood interferes with regular hematopoiesis. This results in a decrease of healthy blood cells, like erythrocytes, thrombocytes and white blood cells, ultimately leading to bone marrow failure (Khwaja et al. 2016). Typical symptoms of AML include fatigue, anaemia, increased risk of infections and bleeding. Although AML can occur at any age, it is most prevalent in elderly people with a median age of 70 years at the time of diagnosis (Juliusson et al. 2009). Increasing age influences treatment success, as up to 40% of all patients under the age of 60 can be cured, while the cure rate drops to 15% in patients older than 60 years (Döhner et al. 2015).

Another important prognostic factor altering the treatment options is the cytogenetic profile of each AML patient. In comparison to CML, which is mostly driven by the *BCR-ABL1* fusion gene (Chereda and Melo 2015), AML is a molecularly heterogeneous disease, characterized by a multitude of mutations and chromosomal aberrations. So far, 5234 driver mutations across 76 genes have been identified in AML, with most patients harboring at least two co-occurring driver mutations (Papaemmanuil et al. 2016). Most frequently occurring somatic mutations in AML are found in genes involved in signal transduction (e.g. *FLT3*, *NRAS*), in epigenetic modifiers (*DNMT3A*, *IDH1*, *IDH2*, *TET2*), in transcription factors (*CEBPA*, *RUNX1*) and in

tumor suppressors (*TP53*, *WT1*) (Kishtagari et al. 2020). Chromosomal translocations resulting in the expression of oncogenic fusion proteins such as AML1-ETO or PML-RARA are also commonly found in AML (Kumar 2011). The identification of genetic abnormalities through molecular diagnostics is crucial to provide the most appropriate treatment regimen for individual AML patients.

1.3. Treatment of AML

Intensive chemotherapy is the standard treatment for most patients diagnosed with AML. Frontline induction chemotherapy is administered in a 7+3 regimen, which consists of a continuous Cytarabine infusion for seven days. This is combined with an anthracycline, most often Daunorubicin, in the first three days of treatment (Dombret and Gardin 2016). Cytarabine is a nucleoside analogon which blocks cell cycle progression from G1 to S phase by inhibiting DNA synthesis (Murphy and Yee 2017). Daunorubicin forms complexes with DNA and inhibits Topoisomerase II leading to single double strand DNA breaks (Murphy and Yee 2017). Both drugs mainly affect rapidly dividing cells, as they undergo mitosis and therefore DNA replication happens more frequently compared to slower growing cells. After completing induction chemotherapy, patients with a high risk of relapse are considered for hematopoietic stem cell transplantation (HSCT), while patients which are not eligible for HSCT are recommended to continue with high dose Cytarabine (HiDAC) as a consolidation therapy (Dombret und Gardin 2016). If neither HiDAC or HSCT are suitable for the patients due to age or co-morbidities, low dose Cytarabine (LDAC) or hypomethylating agents like Azacitidine could be administered to control residual disease (Dombret and Gardin 2016). Recently, the development and approval of several targeted therapies has broadened treatment options.

1.4. Targeted therapies in AML

Targeted therapies block the growth of malignant cells by interfering with specific genes and proteins. In comparison to traditional chemotherapy, which targets all rapidly dividing cells, targeted therapies aim to eliminate cells that are dependent on specific molecular factors, while mostly sparing healthy cells. In general, oncogenic drivers that are found in a substantial number of patients represent promising candidates for targeted therapies. Due to the heterogeneity of oncogenic drivers in AML, distinct treatments for each subgroup are required.

The first identified and so far most effective targeted therapy in AML is all-trans retinoic acid (ATRA) and arsenic trioxide (ATO) in patients with acute promyelocytic leukemia (APL) harboring *PML-RARA* fusions. ATRA and ATO bind to the PML-RARA fusion protein, leading

to its degradation thereby inducing cell death in APL cells (Nasr et al. 2008). Other approved targeted therapies for AML include the small molecule inhibitors Midostaurin and Gilteritinib (targeting FLT3), Ivosidenib and Enasidenib (targeting (IDH1 and IDH2, respectively), the BCL-2 inhibitor Venetoclax and Glasdegib, which inhibits the SMO receptor in the Hedgehog pathway (Short et al. 2020). The antibody-drug conjugate Gemtuzumab ozagamicin also represent a targeted therapy, as it specifically inhibits CD33-positive cells.

The *FLT3* gene is mutated in around 30% of AML patients. Most prominent mutations represent internal tandem duplications (ITD) or point mutations in the tyrosine kinase domain (TKD) (Short et al. 2018). Both types of mutations result in constitutive activation of the FLT3 receptor tyrosine kinase, which leads to increased cellular proliferation (Brandts et al. 2005). Midostaurin is a multi-target kinase inhibitor that is effective against both *FLT3*-ITD and TKD mutations and has additional activity against multiple other kinases. Gilteritinib is a second-generation FLT3 inhibitor and is more specific to mutant *FLT3*. Both compounds are approved for therapy of AML in patients harboring *FLT3* mutations (Short et al. 2020).

Other promising therapeutic targets are mutated forms of Isocitrate dehydrogenase 1 (IDH1) or 2 (IDH2). Mutations in the *IDH1* or *IDH2* genes occur in up to 15% and up to 20% of AML patients, respectively (Short et al. 2018). These mutations lead to the accumulation of the oncometabolite 2-hydroxyglutarate (2-HG). As 2-HG inhibits DNA demethylases, *IDH1/2* mutations result in a hypermethylated state of DNA, which can activate oncogenes and inactivate tumor suppressors. Ivosidenib and Enasidenib – which inhibit mutated IDH1 and IDH2 respectively – are so far the only approved IDH inhibitors and have shown efficacy in a significant number of patients (Short et al. 2020).

In contrast to FLT3 and IDH1/2, where specific mutations drive the disease and are therefore attractive direct targets for treatment, BCL-2 is not an oncogenic driver, but important for regulating the intrinsic pathway of apoptosis. BCL-2 overexpression was found in many hematological malignancies (Roberts and Huang 2017), including AML, which depends on BCL-2 for cell survival. In spite of the efficacy of the BCL-2 inhibitor Venetoclax in many patients, there is a lack of clear prognostic markers for the identification of AML patients who would respond best to Venetoclax treatment (DiNardo et al. 2020).

A different approach to treat AML is the targeting of self-renewal mechanisms in leukemic cells by inhibiting the hedgehog signaling pathway with the Smoothened (SMO) inhibitor Glasdegib. Currently, the efficacy of many different drugs targeting cell cycle kinases, mutated *TP53*,

surface antigens, the RAS pathway and different apoptotic proteins in AML is evaluated in multiple clinical trials (Short et al. 2020). Despite the efficacy of many targeted drugs in AML, they are mainly used in patients who are not fit enough for standard induction chemotherapy or in relapsed patients. To improve therapeutic outcomes for all patients independent of age or condition, the identification of clear prognostic markers and the development of rational combinations of targeted therapies with chemotherapy or other targeted therapies is required.

1.5. The B-cell-lymphoma 2 (BCL-2) family proteins

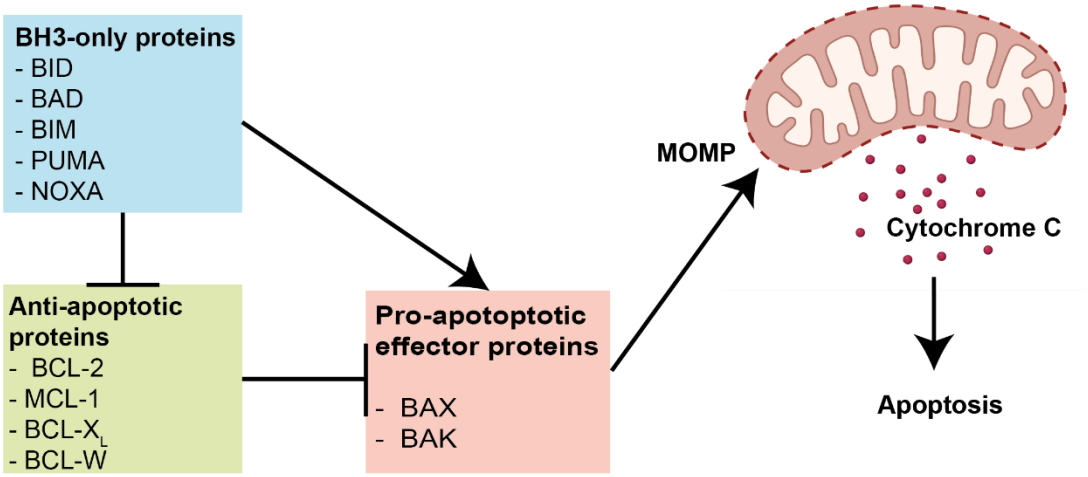


Figure 1: Regulation of the intrinsic apoptotic pathway by BCL-2 family proteins. Anti-apoptotic proteins bind to pro-apoptotic effectors to inhibit them. Pro apoptotic initiators can bind to anti-apoptotic proteins and replace effectors, or they can activate effectors directly. If effectors are not bound to anti-apoptotic proteins, they oligomerize and form pores in the mitochondrial outer membrane (Mitochondrial outer membrane permeabilization (MOMP)), leading to apoptosis via cytochrome C release and Caspase activation.

The BCL-2 family consists of 25 members which either promote or inhibit apoptosis (Youle and Strasser 2008). In healthy cells, there is a balance of the activity between pro- and anti-apoptotic proteins. External stimuli such as stress or withdrawal of growth factors can tip the balance in favor of pro-apoptotic factors. Anti-apoptotic proteins (e.g. BCL-2, BCL-X_L, MCL-1) inhibit pro-apoptotic effector proteins (e.g. BAX, BAK). When pro-apoptotic effectors are released from anti-apoptotic proteins, they oligomerize at the mitochondrial outer membrane and form pores, which leads to the release of cytochrome C from the mitochondria, Caspase activation and ultimately apoptosis (Fig. 1) (Youle and Strasser 2008). A third group of BCL-2

proteins - the BH3-only sensitizers (e.g. BIM, NOXA, PUMA) – promote apoptosis either indirectly by inhibiting anti-apoptotic proteins (Willis 2008) or directly by activating pro-apoptotic proteins (Fig. 1) (Youle 2007). High levels of BCL-2 have been observed to contribute to resistance to apoptosis and chemoresistance in CLL (Pepper et al. 1998), which sparked interest in developing specific BCL-2 inhibitors.

1.6. Targeting anti-apoptotic proteins

Pro-survival proteins can be targeted by compounds that bind to the hydrophobic groove in BCL-2 family proteins that is normally occupied by BH3-only proteins (Merino et al. 2018). Hence these compounds are called BH3-mimetics. The first and so far only FDA-approved BH3-mimetic is Venetoclax, an orally available selective BCL-2 inhibitor (Deeks 2016). Venetoclax is approved for the treatment of CLL, small lymphocytic lymphoma (SLL) and AML. The approval of Venetoclax in AML therapy provides a novel treatment option for elderly patients who are unfit for induction chemotherapy (DiNardo et al. 2019). In these cases, Venetoclax is widely administered in combination with Azacitidine, Decitabine, or LDAC.

Currently many different clinical trials investigate the efficacy of Venetoclax in AML as monotherapy, in combination with chemotherapy or in combination with a variety of other targeted agents against FLT3, MCL-1, and IDH1/2 (Samra et al. 2020). As the success of Venetoclax in clinical studies proved that BCL-2 is an actionable target in AML, many more small molecules targeting other pro-survival proteins have been developed and are currently undergoing preclinical and clinical evaluation. Examples of such compounds are the MCL-1 inhibitors AZD-5991 and S63845 (Bolomsky et al. 2020) or the BCL-2/BCL-X_L inhibitor AZD-4320 (Balachander et al. 2020).

1.7. Resistance to targeted therapy in AML

Despite the efficacy and few side effects of targeted therapies, cancer cells often develop resistance. Eventually, this results in therapeutic failure and if possible, the treatment has to be changed. For instance, the switching from mutant IDH1 to IDH2 confers resistance to Ivosidenib (Harding et al. 2018). Secondary point mutations in the *FLT3* TKD lead to resistance to some FLT3 inhibitors (Daver et al. 2015). The upregulation of the RAS–RAF–MEK– ERK pathway is another resistance mechanism to FLT3 inhibition (McMahon et al. 2019).

The variety of molecular mechanisms leading to Venetoclax resistance illustrates how different cellular pathways can result in resistance to the same small molecule. The affinity of Venetoclax for the BCL-2 protein can be reduced by the Gly101Val mutation in BCL-2

(Thangavadivel and Byrd 2019). Alternatively, a switch in expression of BCL-2 proteins, such as up-regulation of MCL-1 or BCL-X_L, confers Venetoclax resistance in AML. Additionally, genome wide CRISPR screens have identified that loss of *BAX* or *TP53* (Nechiporuk et al. 2019) and overexpression of the *OPA1* Mitochondrial Dynamin like GTPase (Chen et al. 2019) can drive Venetoclax resistance. Furthermore, reduced mitochondrial apoptotic priming is another resistance mechanism for BH3-mimetics targeting BCL-2 or MCL-1 (Bhatt et al. 2020).

So far, the majority of described resistance mechanisms to targeted therapies are based on mutations or changes in gene expression. However, it has long been known that changes in pharmacokinetics can influence drug response as well, either by decreased cellular drug intake or by increased drug efflux (Gottesman et al. 2002). Whether increased drug efflux contributes to resistance to recently approved targeted therapies is an ongoing research question.

1.8. ATP-binding cassette transporters

ATP-binding cassette (ABC) transporters are a family of transmembrane proteins which are able to transport substrates across membranes. ABC transporters harbor two characteristic hydrophilic nucleotide binding domains (NBD) that are needed to bind and hydrolyze ATP and one to three hydrophobic membrane spanning domains (MSD) that are required for substrate recognition and translocation. Substrates of ABC transporters include lipids, hormones, ions, nucleosides, metabolites and xenobiotics. The transport function of ABC transporters is essential for many aspects of cell physiology, including detoxification, metabolism and cell signaling.

In humans, 48 ABC transporters have been identified. They are categorized into 7 subgroups (ABCA-ABCG), which differ in structure, function and tissue expression (Vasiliou et al. 2009). Due to their important physiological functions, mutations in genes coding for ABC transporters can lead to severe genetic diseases (Gottesman and Ambudkar 2001), with cystic fibrosis (*ABCC7*) being the most prominent (Ratjen et al. 2015). Other diseases caused by mutations in ABC transporters include Stargardt disease (*ABCA4*) (Cremers et al. 2020), Tangier disease (*ABCA1*) (Rust et al. 1999) and the Dubin–Johnson syndrome (*ABCC2*) (Toh et al. 1999).

As some ABC transporters can efflux anti-cancer drugs, their overexpression has been associated with a poor response to chemotherapy and multi drug resistance (MDR) (Xiao et al. 2021). ABCB1, ABCC1 and ABCG2 are the most prominently studied ABC transporters in MDR in cancer, but at least 16 other ABC transporters have the potential to efflux anticancer drugs (Fletcher et al. 2010). Despite ABC transporters being a promising target in improving

efficacy of chemotherapy, there are currently no approved inhibitors of ABC transporters. Clinical trials of ABCB1 inhibitors not just failed due to toxic side effects caused by unspecific inhibition of multiple ABC transporters, but also due to poor study design (Robey et al. 2018).

1.9. ABCC1 (Multiple Resistance Associated Protein 1, MRP1)

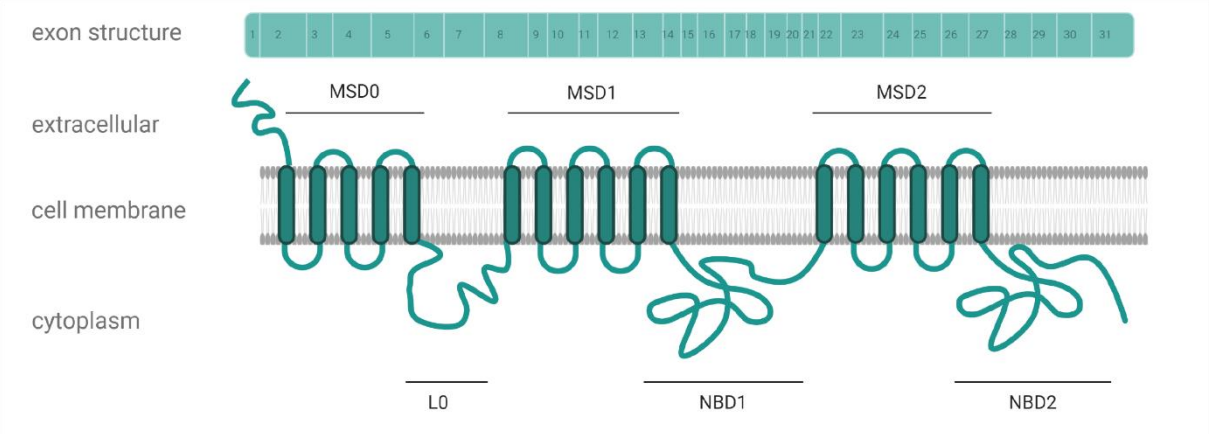


Figure 2: Topological structure of ABCC1. ABCC1 consists of the three membrane spanning domains (MSD0-MSD2), a linker region (L0) that connects MSD0 to MSD1 and two nucleotide binding domains (NBD1, NBD2). The corresponding exons and the cellular location of the respective domains is indicated.

ABCC1 is a member of the Multiple Resistance Associated Protein (MRP) subfamily and was first identified in a Doxorubicin resistant small lung cancer cell line (Cole et al. 1992). ABCC1 is expressed in the blood-brain barrier, lung, testis, kidney, peripheral blood mononuclear cells, skeletal and cardiac muscle, and placenta (He et al. 2012). Unlike other ABC transporters - which are mainly located at the apical site of membranes - ABCC1 is found at the basolateral side of the plasma membrane (Evers et al. 1996). Distinct from other ABC transporters but in common with ABCC2, ABCC3, ABCC6 and ABCC10, ABCC1 has a third membrane spanning domain (MSD0) at the N-terminus, which is connected to the MSD1 via a linker domain (L0) (Fig. 2). While it has been postulated that MSD0 is important for subcellular localization of the protein, the exact function of this domain is not known (Westlake et al. 2005). Point mutations in MSD0 impair transporter function (Ito et al. 2003), but complete loss of MSD0 results in a transporter molecule that is still capable of translocating substrates (Bakos et al. 2000).

ABCC1 is a multispecific transporter which mainly transports organic anions and conjugated substrates (a selection of the most relevant xenobiotic and physiological ABCC1 substrates is listed in Table 1). The best characterized endogenous ABCC1 substrates are leukotriene C_4

(LTC_4) and oxidized glutathione (glutathione disulfide, GSSG). Transport of glutathione (GSH) and GSSG is an unique feature of several members of the ABCC subfamily that distinguishes it from other ABC transporter subfamilies (Ballatori et al. 2005, Kruh et al. 2006). ABCC1-mediated transport of LTC_4 contributes to the inflammatory response (Wijnholds et al. 1997). GSH and GSSG efflux mediated by ABCC1 affect many biological processes like redox homeostasis, metabolism and detoxification (Cole 2014a), and glutathione itself is an important co-factor in ABCC1-mediated transport of various substrates. *ABCC1*-knockout cells have high intracellular GSH levels, while cells overexpressing *ABCC1* have lower levels of intracellular GSH (Laberge et al. 2007).

Table 1: Selected xenobiotic and physiological substrates of ABCC1. Data extracted from (Cole 2014b, Kunická and Souček 2014)

Xenobiotics	**Physiological substrates**
Anticancer drugs:	● Glutathione (GSH, GSSG)
● Vinca alkaloids (Vinblastine, Vincristine)	● Leukotrienes C_4, D_4 and E_4
● Anthracyclines (Daunorubicin, Doxorubicin)	● Prostaglandin A_2
● Epipodophyllotoxins (Etoposide, Teniposide)	● Hydroxynonenal-GSH conjugate
● Camptothecins (Topotecan, Irinotecan)	● Estrone-3-sulphate
● Methotrexate	● Dehydroepiandrosterone-3-sulfate
● Antiandrogens (Flutamide)	● Sulfatolitocholyl taurine
● Taxanes (Paclitaxel, Docetaxel)	● Bilirubin
● Kinase inhibitors (Gefinitinib, Imatinib)	● Glucuronosylbilirubin
HIV protease inhibitors:	● Estradiol-17-b-D-glucuronide
● Ritonavir, Saquinavir	● Folic acid
Antibiotics:	● L-Leucovorin
● Difloxacine, Grepafloxacine	
Toxins:	
● Aflatoxin B1	
Pesticides:	
● Fenitrothion, Methoxychlor	

Due to its ability to transport many anticancer agents, overexpression of ABCC1 has been implicated in resistance to chemotherapy. In addition, high expression of ABCC1 has been

associated with adverse prognosis in some cancer types (Haber et al. 2006, Walsh et al. 2010). The most commonly used ABCC1 inhibitors are the pyrazolopyrimidine Reversan and the leukotriene D4 receptor antagonist MK-571 (Cole 2014b). Reversan was identified in a drug screen for ABCC1 inhibitors and increased efficacy of chemotherapy in preclinical neuroblastoma models (Burkhart et al. 2009). However, these drugs have not been optimized for *in vivo* use and can thus only be used in preclinical experiments using cultured cells.

1.10. Glutathione (GSH)

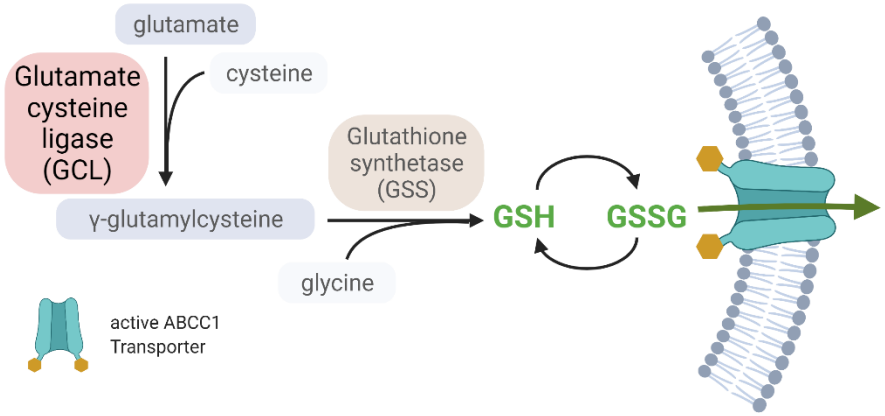


Figure 3: Synthesis of Glutathione and efflux of GSSG via ABCC1. The enzyme Glutamate-cysteine ligase (GCL) combines glutamate and cysteine to produce gamma-glutamylcysteine. Then, the enzyme Glutathione synthetase (GSS) adds glycine to the C-terminus of gamma-glutamylcysteine to form reduced glutathione (GSH), which can be oxidized to GSSG and effluxed via ABCC1

GSH is the most abundant intracellular non-protein thiol that protects cells from oxidative stress. Reduced GSH can neutralize reactive oxygen species (ROS), leading to the formation of GSSG, which can be reduced again by GSSG reductase or effluxed via ABCC1 (Fig. 3) (Lu 2009). ABCC1 can transport reduced GSH as well, even though at a lower affinity compared to GSSG (Mueller et al. 2005).

The ratio of reduced versus oxidized GSH (GSH/GSSG) is an indicator of oxidative stress, as high levels of reduced GSH are needed to maintain cellular redox homeostasis. In addition to its cytoprotective role against ROS, GSH is needed for detoxification of metabolites and

xenobiotics, cysteine metabolism, protein synthesis and cell cycle regulation (Hammond et al. 2001).

GSH is synthesized from two amino acids, glutamate and L-cysteine, in two ATP-dependent enzymatic reactions (Fig. 3): First, the enzyme Glutamate-cysteine ligase (GCL) combines the two amino acids to produce gamma-glutamylcysteine. Second, the enzyme Glutathione synthetase (GSS) adds glycine to the C-terminus of gamma-glutamylcysteine, which results in the formation of reduced GSH (Lu 2013). The GCL inhibitor Buthionine Sulfoximine (BSO) can be used to pharmacologically inhibit GSH synthesis (Griffith and Meister 1979). In addition to being a substrate of ABCC1, GSH has a key role in ABCC1-mediated efflux. On the one hand, GSH-conjugated substances are transported mainly by ABCC1 (GSH-coupled transport, Fig. 4b). On the other hand, some drugs are not conjugated to GSH, but require co-transport of GSH to be exported by ABCC1 (Co-Transport, Fig. 4a) (Cole and Deeley 2006).

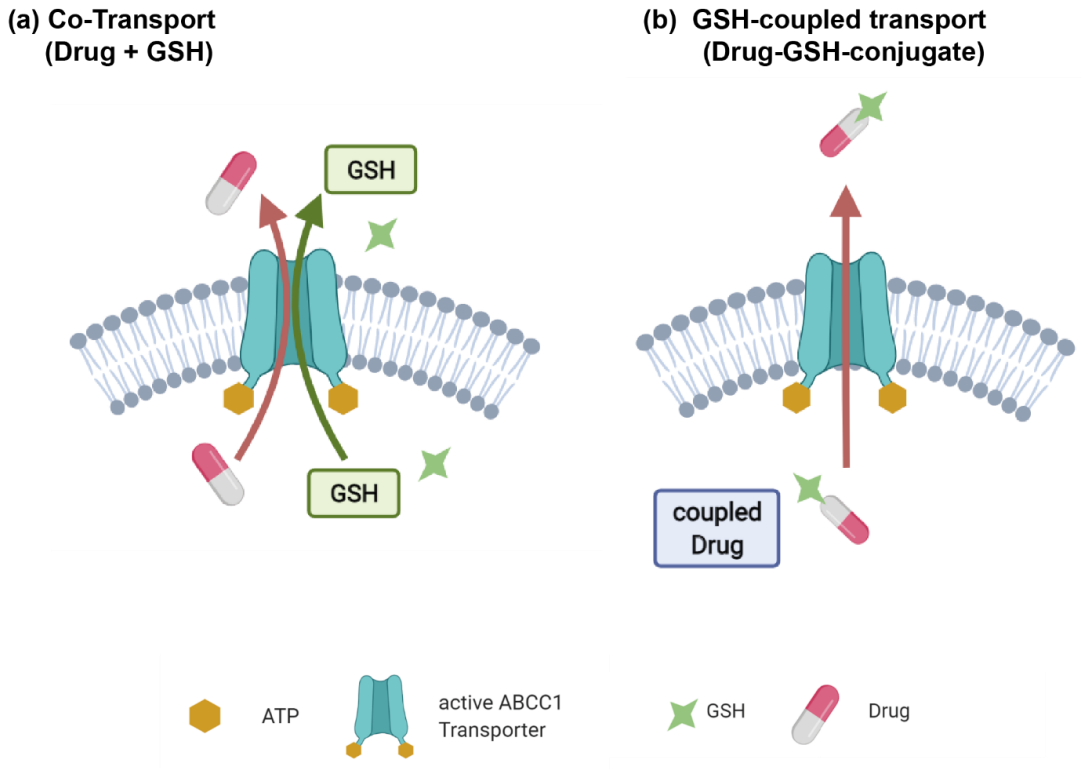


Figure 4: GSH-dependent drug efflux via ABCC1. a) ABCC1-mediated co-transport of drugs in the presence of glutathione (GSH), b) Efflux of glutathionylated drugs (GSH conjugates) via ABCC1.

1.11. Glutathione-S-transferases (GST)

Glutathione S-Transferases (GST) are enzymes that conjugate GSH to xenobiotics in a first step of detoxification (Fig. 5). In most instances, glutathionylation makes compounds less reactive and thereby less toxic to cells (Hayes et al. 2005, Oakley 2011). The conjugation of xenobiotics with GSH also renders many of those substances more polar, which makes them better substrates for active transport by ABC Transporters such as ABCC1 and ABCC2 (Fig. 4b and Hayes et al. 2005). Expectedly, overexpression of *ABCC1* is often accompanied by the simultaneous overexpression of GSTs (Bansal und Celeste Simon 2018). The most commonly overexpressed GSTs in cancer are *GSTA1*, *GSTM1*, *GSTO1-1* and *GSTP1* (Pljesa-Ercegovac et al. 2018).

In addition to contributing to cellular detoxification and thereby potentially to chemotherapy resistance, GSTs can also modulate the activity of Mitogen activated protein kinases (MAPK) (Laborde 2010). Due to their role in detoxification as well as cell signaling, the use of GST inhibitors in improving cancer therapy is under investigation. For instance, treatment with Ethacraplatin – a conjugate of cisplatin and the GST inhibitor ethacrynic acid – is able to overcome cisplatin resistance in preclinical cancer models (Li et al. 2017). Another GST inhibitor, the GSH-peptidomimetic Ezatiostat, has also been found to potentiate the effect of a variety of different anti-cancer drugs like Chlorambucil, Doxorubicin and Melphalam *in vitro* (Zhang et al. 2021). Moreover, Ezatiostat has a stimulatory effect on hematopoiesis, which has led to clinical studies investigating its efficacy for the treatment of myelodysplastic syndrome (Zhang et al. 2021). Other commonly used GST inhibitors to enhance cytotoxic properties of chemotherapeutics are NBDHEX, MI175, KT53, Piperlongumine and Curcumin (Singh and Reindl 2021).

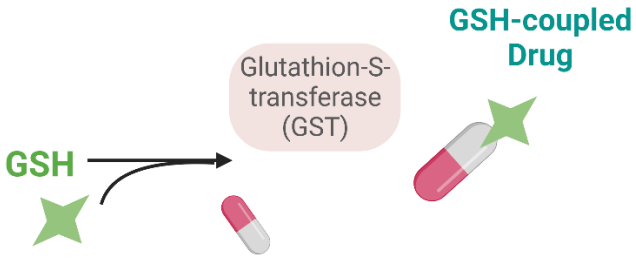


Figure 5: Schematic illustration of the conjugation of GSH to a drug by a glutathione-S-transferase. Reduced glutathione (GSH) is conjugated to a drug by Glutathione-S-transferases (GSTs) thereby rendering them less effective and easing further metabolic processes or efflux.

1.12. Aim of this study

While several ABC transporters are well known to mediate resistance to a variety of frequently used cytostatic drugs, it is not known whether ABC transporters can modulate the sensitivity to newly approved targeted therapies. Especially in AML, where the recent approval of different targeted drugs has greatly expanded treatment options, characterizing the influence of ABC transporters on these new drugs can help improve patient management. Thus, we aimed to systematically evaluate the role of ABC transporters in the response to targeted therapies in AML.

As a result of this investigation, a CRISPR/Cas9 loss of function screen of all 48 ABC transporters in Cas9-expressing MOLM-13 cells identified that loss of *ABCC1* potentiated the efficacy of the BCL-2 inhibitor Venetoclax (unpublished). The aim of this work was to further investigate the influence of ABCC1 inhibition on the response to BCL-2 inhibitors in human AML cell lines. By using the CRISPR/Cas9 system and shRNA mediated knockdown we studied the effect of *ABCC1* loss in different cell lines. Additionally, the use of the ABCC1 inhibitors MK-571 and Reversan allowed us to pharmacologically modulate ABCC1 function. Furthermore, we investigated the role of GSH, an important factor in ABCC1-mediated drug efflux by pharmacologically inhibiting the GSH synthesis and GSTs in combinatorial treatment with the BCL-2 inhibitors Venetoclax and AZD-4320. Finally, to examine the patient relevance of our findings, we analyzed the expression of multiple ABC transporters in primary patient-derived AML cells and treated primary human AML cells with BCL-2 inhibitors in combination with the ABCC1 inhibitor Reversan.

2. Materials and Methods

2.1. Cell culture

Stable Cas9-expressing clones of HL-60 [*MYC* amplification], MOLM-13 [*FLT3*-ITD; *MLL-AF9*] and THP-1 [NRAS mut.; *MLL-AF9*] were used for this study. The MV4-11 [cell line *FLT3*-ITD; *MLL-AF4*] used in this work does not express Cas9. All AML suspension cell lines (HL-60, MOLM-13, MV4-11, THP-1) were kept in RPMI 1640 (Gibco, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, USA), 1% Penicillin/Streptomycin (Gibco, USA), 2% L-Glutamine (Gibco, USA), 1% Sodium pyruvate (Sigma-Aldrich), 0.1% 2-Mercaptoethanol (Gibco, USA) and 2% HEPES (Sigma-Aldrich, USA) at 37°C, 5% CO_2, and 95% humidity. Cells were split every 2-3 days and adjusted to $5*10^5$ cells/ml. Primary patient-derived AML cells were cultivated in IMDM (Thermo Fisher Scientific, USA) supplemented with 15% BIT 9500 Serum Substitute (STEMCELL Technologies, Canada), 100 ng/ml SCF (ImmunoTools, Germany), 50 ng/ml FLT3L (ImmunoTools, Germany), 20 ng/ml IL-3 (ImmunoTools, Germany), 20 ng/ml G-CSF (ImmunoTools, Germany), 0.1 mM 2-Mercaptoethanol (Gibco, USA), 50 µg/ml Gentamicin (Thermo Fisher Scientific, USA), 10 µg/ml Ciproflaxin (Thermo Fisher Scientific, USA), 500 nM SR1 (APExBIO, USA) and 1 µM UM279 (APExBIO, USA) at 37°C, 5% CO_2, and 95% humidity. LentiX cells, which are used for virus production, were cultivated in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, USA), 1% Penicillin/Streptomycin (Gibco, USA) and 2% L-Glutamine (Gibco, USA).

2.2. Plasmids and cloning

2.2.1. Single guide RNA (sgRNA) plasmid cloning

The lentiviral backbone pLenti-hU6-sgRNA-IT-PGK-iRFP670 was digested with the restriction enzyme BsmBI (NEB, USA) and dephosphorylated with Antarctic phosphatase (NEB, USA). The digested and dephosphorylated plasmid was loaded on an 0.7% agarose gel, the linearized plasmid was excised and purified using the MiniPex 3 in 1 Kit (IMP, Austria). The forward (F) and reverse (R) oligos of the sgRNA sequences were phosphorylated and annealed using1 µl oligo F (100 µM), 1 µl oligo R (100 µM), 1 µl 10X T4 Ligation Buffer with ATP (NEB, USA), 6.5 µl ddH_2O, 0.5 µL T4 Polynucleotide Kinase (NEB, USA) for 30 minutes at 37°C in a thermocycler. The annealed oligos were diluted 1:200. For the ligation reaction, 1 µl of the annealed and diluted oligos, 1 µL T4 Ligase Buffer (NEB, USA),1 µl T4 DNA Ligase (NEB, USA), 50 ng of the digested lentiviral plasmid and ddH_2O were added to a total reaction volume of 10 µl and incubated at room temperature for 10 minutes. The ligated plasmid was

transformed into NEB Stable Competent E. coli (High Efficiency) (NEB, USA) and plasmids were purified using the MiniPex 3 in 1 Kit (IMP, Austria).

2.2.2 Short hairpin RNA (shRNA) cloning

The shRNA target sequence (0.05 ng) was PCR amplified with 25 µL of Phusion® High-Fidelity PCR Master Mix (NEB, USA) and 2.5 µl of each primer, mirF-oligo-forward (10 µM) and mirF-oligo-reverse (10 µM), in a total reaction volume of 50 µl under standard PCR conditions. PCR products were purified using the MiniPex 3 in 1 Kit (IMP, Austria). 25 µl of the purified PCR product was digested with 2 µl XhoI (NEB, USA), 1 µl Ecor1- HF (NEB, USA), 3.5 µL CutSmart Buffer (NEB, USA) and 3.5 µL ddH_2O for 3 h at 37°C in a total reaction volume of 35 µl. The reaction product was loaded onto a 2% agarose gel and the digested product was excised and gel purified with the MiniPex 3 in 1 Kit (IMP, Austria). The lentiviral shRNA expression vector SFFV-iRFP670-mirF-NeoR was digested with XhoI (NEB, USA). For the ligation, 300 ng digested backbone, 10 ng digested PCR product, 1 µl 10x T4 buffer and 0.5 µL T4-ligase were incubated for 15 minutes at room temperature in a total reaction volume of 10 µL. The ligation product was transformed into NEB Stable Competent E. coli (High Efficiency) (NEB, USA) and plasmids were purified using the MiniPex 3 in 1 Kit (IMP, Austria).

2.2.3 TA cloning

Identification of the exact sequence changes of ABCC1 knockout clones was performed by using the TA Cloning® Kit (Thermo Fisher Scientific, USA). For the ligation, 2 µL of the linearized pCR®2.1 vector, 0.5 µL purified PCR product of the target region of the respective sgRNAs targeting *ABCC1* in knockout clones, 2 µl 5X T4 DNA ligase reaction buffer, 5 µL ddH_2O and 1 µL ExpressLinkTM T4 DNA Ligase (5 units) were incubated together for 15 minutes. The ligation product was transformed into NEB Stable Competent E. coli (High Efficiency) (NEB, USA) and plasmids were purified using the MiniPex 3 in 1 Kit (IMP, Austria).

2.3. Bacterial transformation

NEB Stable Competent E. coli (High Efficiency) (NEB, USA) were thawed on ice and 2-5 µL of DNA was added. After 20 minutes of incubation on ice, bacteria were heat-shocked at 42°C for 45 seconds followed by a 2 minute incubation on ice. Then, 950 µL of LB-media (Carl Roth, Germany) was added and the mixture was incubated at 37°C for 1 hour at 650 rpm on a shaking incubator. Bacteria were then streaked on LB-Agar (Carl Roth, Germany) plates containing 100 µg/mL Carbenicillin (Carl Roth, Germany) and incubated overnight at 37°C. For TA cloning, bacteria were plated on agar plates containing X-Gal (Merck, Germany) and IPTG

(Merck, Germany) to allow blue-white screening of colonies. Single colonies were picked the next day and incubated in 5 ml LB-media supplemented with 100 µg/mL Carbenicillin (Carl Roth, Germany) overnight at 37°C in a shaking incubator. Bacteria were then pelleted and plasmids isolated using the MiniPex 3 in 1 Kit (IMP, Austria). Sanger sequencing was use used to confirm the presence of desired sequences in isolated plasmids (Microsynth, Switzerland).

2.4. Lentiviral transduction

LentiX cells were seeded at a confluency of 50-70% in 6-well plates and transfected with the respective constructs (0.7 µg) together with the packaging plasmids psPAX2 (0.3 µg) and pMD2.G (0.2 µg) (Addgene, USA) using Polyethylenimine (Polysciences, USA). 24 hours later, medium was exchanged to RPMI 1640 (Gibco, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, USA), 1% Penicillin/Streptomycin (Gibco, USA), 2% L-Glutamine (Gibco, USA), 1% Sodium pyruvate (Sigma-Aldrich), 0.1% 2-Mercaptoethanol (Gibco, USA) and 2% HEPES (Sigma-Aldrich, USA). Supernatants were collected and filtered through 0.45 µm filters (TPP, Switzerland) 48 and 72 hours after transfection and stored at 4°C until further use. For transduction of target cells, filtered supernatant containing lentiviral particles was added in a ratio of 1:5 - 1:10 to $1*10^6$ cells in a final volume of 1 ml, supplemented with 10 µg/ml Polybrene (Merck, Germany). Cells were spinoculated at 1000xg for 90 minutes at room temperature. 24 hours after spinoculation, the supernatant was replaced with fresh, virus free media.

2.5. Competitive cell proliferation assays

After transduction, cells were seeded at a density of $4*10^5$ cells/ml in 24 well plates (Greiner Bio One, Germany) in triplicates per condition. To assess the effect of *ABCC1* knockout and knockdown, levels of IRFP670+ cells were measured every 2-3 days by flow cytometry on an IntelliCyt IQueScreener Plus (Sartorius, Germany). Levels of IRFP670+ cells were normalized to day 3 after lentiviral transduction. In case of drug treatment, normalization was performed to values obtained on the first day of drug treatment and to the respective DMSO control.

2.6. Growth curves

Cells were seeded at a density of $4*10^5$ cells/ml in 24-well plates (Greiner Bio One, Germany) and treated with either DMSO as control or with the respective compounds in the indicated concentration in triplicates. Cells were counted every 2-3 days on an IntelliCyt IQueScreener Plus instrument (Sartorius, Germany) to calculate growth rate and cumulative cell numbers, using Microsoft Excel. Cells were split in regular intervals to re-adjust cell numbers to $4*10^5$

cells/ml and treated with DMSO or the respective compound supplemented to the freshly added media. Growth rate was determined as: $\frac{Cell\ number\ t(1)}{Cell\ number\ t(0)} * splitting\ factor.$

2.7 Compounds

Table 2: List of all compounds used in this study

Compound	Target	Provider
Venetoclax	BCL-2	MedChemExpress (USA)
AZD-4320	BCL-2, BCL-X_L	MedChemExpress (USA)
AZD-5991	MCL-1	MedChemExpress (USA)
Midostaurin	Multiple Tyrosine Kinases	Selleck Chemicals (USA
Gilteritinib	FLT3	Selleck Chemicals (USA)
Etoposide	Topoisomerase II	MedChemExpress (USA)
Reversan	ABCC1	Merck (Germany)
MK-571	ABCC1	MedChemExpress (USA)
Buthionine Sulfoximine (BSO)	Glutamate Cystein Ligase (GCL)	Merck (Germany)
Ethacrynic acid	Glutathione S-transferases (GST)	Sigma-Aldrich (USA)
Ezatiostat (TLK-199)	Glutathione S-transferases (GST)	MedChemExpress (USA)

2.8. Cell viability assays

Cells were seeded at a density of $5*10^3$ cells per 100 µL media per well in white 96-well plates (Thermo Fisher Scientific, USA) and treated with a dilution series of 9 different drug concentrations + media in triplicates for each condition. For combinatorial treatments with ABCC1 inhibitors Reversan and MK-571 the medium was supplemented with the indicated concentrations of the respective compounds. To impair transporter function before the addition of other drugs, cells were pre-treated for 24 h with Reversan or MK-571 before other drugs were added. After 5 days of incubation cell viability was measured with the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, USA) according to the manufacturer's instructions on a Spark® multimode microplate reader (TECAN, Switzerland). Dose–response curves with IC_{50} values were generated using the Prism 6.0.1 (GraphPad, USA) software. In experiments

with primary patient derived cells, $1*10^4$ cells per well were seeded and viability was measured after 3 days of incubation.

2.9. Genotyping

$5*10^6$ cells were spun down at 300xg, washed twice with PBS, pellets were shock frozen in liquid nitrogen and stored at -80°C until further use. Genomic DNA was extracted using the Quick gDNA Miniprep Kit (ZYMO Research, USA). Target regions of the respective sgRNAs targeting ABCC1 were amplified with PCR using LA Taq DNA Polymerase (Takara Bio, Japan). PCR amplification was conducted in a 50 µl reaction consisting of 15.5 µl ddH_2O, 5 µL 10X LA PCR™ Buffer II (Mg^{2+} free), 5 µL 25 mM $MgCl_2$, 8 µL dNTP mixture, 5 µL of 10 µM forward primer, 5 µl of 10 µM reverse primer, 5 µl DMSO, 1 µl of 100 ng/µl DNA template and 0.5 µL TaKaRa LA Taq™ (5 units/µl) on a C1000 Touch Thermal Cycler (Bio-Rad, USA) with 30 cycles (10 seconds denaturation at 98°C, 1 minute annealing at 56-59°C, depending on the primer pair used, and 1 minute elongation at 72°C). The PCR products were loaded onto a 1.5% agarose gel to check for correct size of the amplicon. PCR products were purified with the MiniPex 3 in 1 Kit (IMP, Austria) and analyzed via Sanger sequencing (Microsynth, Switzerland). Tracking of Indels by Decomposition (TIDE) (Brinkman et al. 2014) analysis was performed to assess the efficiency of CRISPR/Cas9 mediated mutagenesis and to identify the type of insertions and/or deletions.

2.10. RT-qPCR

$5*10^6$ cells were spun down at 300xg, washed twice with PBS, pellets were shock frozen in liquid nitrogen and stored at -80°C until further use. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using the RevertAid first-strand cDNA synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol on a C1000 Touch Thermal Cycler (Bio-Rad, USA). Per reaction, 1000 µg of total RNA was used for cell lines. For patient samples the amount of total RNA per reaction was downscaled to 100 µg. For RT-qPCR, cDNA was amplified in triplicates for each examined gene on the Bio-Rad CFX96-Real-Time PCR Detection System (Bio-Rad, USA) using the SsoAdvanced Universal SYBR green Supermix (Bio-Rad, USA). Relative expression levels were determined by normalizing C(t) values to human β-ACTIN using the 2-ΔΔC(t) method. Absolute gene expression was determined by calculating the percentage of gene expression relative to human *β-ACTIN* using the formula $2^{(-\Delta C(t))} * 100$.

2.11. Data analysis and statistics

Experiments were performed in duplicates or triplicates and data is shown as mean ± SD. Statistical analysis was performed using Prism 6.0.1 (GraphPad, USA). P-values were calculated using the unpaired students T-test, results were considered significant when $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, n.s. = not significant).

3. Results

Previous findings of our group identified ABCC1 as a potential mediator of the response of AML cells to Venetoclax (unpublished). This work aimed to further clarify the role of ABCC1 in the response to targeted therapies. Using CRISPR/Cas9 mediated mutagenesis of *ABCC1* and shRNA mediated knockdown of *ABCC1* in human AML cell lines, we were able to study the effect of loss or reduction of *ABCC1* expression in drug-naïve as well as drug-treated cells. The use of different compounds to modulate either ABCC1 function or important factors in ABCC1 mediated transport (GSH and GSTs) allowed us to further determine whether targeted agents could be substrates of ABCC1. Moreover, the analysis of ABC transporter expression in primary human AML cells provided insights into the most relevant ABC transporters in patients and how their expression is associated with the outcome of Venetoclax treatment. All results are described in the following sections and contribute to clarification of the role of ABCC1 in modulating drug response to targeted therapies, especially BCL-2 inhibitors.

3.1. Drug sensitivity of human AML cell lines

The first step of this work was to determine appropriate concentration ranges of each drug for subsequent experiments. We generated dose response curves and calculated IC_{50} values (Table 3) of a variety of compounds for four commonly used AML cell lines (HL-60, MOLM-13, MV4-11, THP-1). In general, these cell lines tolerated high doses of the Glutamate cysteine ligase (GCL) inhibitor Buthionine Sulfoximine (BSO), to the Glutathione S-transferases (GST) inhibitors Ethacrynic acid and Ezatiostat and to the ABCC1 inhibitors MK-571 and Reversan. On the other hand, their sensitivity to the targeted drugs against BCL-2 (Venetoclax and AZD-4320), MCL-1(AZD-5991) and FLT3 (Midostaurin) was very heterogenous. The THP-1 cell line was the most resistant cell line to these compounds, whereas MOLM-13 and MV4-11 cell lines were very sensitive, with IC_{50} values in the low nanomolar range. HL-60 cells showed an intermediate sensitivity to BCL-2 inhibitors, with IC_{50} values ranging from 200-100 nM. All cell lines showed a similar sensitivity to the Topoisomerase II inhibitor Etoposide, with IC_{50} values ranging from 100-200 nM.

Table 3: IC_{50} [nM] values of all the compounds and cell lines used, values are representative of multiple independently performed dose response curves.

	HL-60	MOLM-13	MV4-11	THP-1
Venetoclax (BCL-2)	500	5	2.5	5000
AZD-4320 (BCL-2, BCL-X_L	200	2	1.2	5000
AZD-5991 (MCL-1)	1000	148	50	n.d.
Etoposide (Topoisomerase II)	130	106	n.d.	110
Midostaurin (FLT3)	254	15	n.d.	n.d.
MK-571 (ABCC1)	>10000	>10000	>10000	>10000
Reversan (ABCC1)	>10000	>10000	>10000	>10000
Buthionine Sulfoximine (BSO) (GCL)	>10000	>10000	>10000	>10000
Ezatiostat (GSTs)	>10000	>10000	n.d.	n.d.
Ethacrynate (GSTs)	>10000	>10000	>10000	>10000

Based on these IC_{50} values, optimal concentration ranges were chosen for the subsequent assays for each cell line. In order to influence cell growth and cellular fitness as little as possible, cells were cultured in media supplemented with indicated drugs in a concentration corresponding to the respective IC_{10} or IC_{20} values in growth curves and competitive proliferation assays.

3.2. Perturbation of *ABCC1* sensitizes AML cell lines to BCL-2 inhibition

To investigate the role of ABCC1 in the normal physiology of AML cells as well as in the presence of anti-cancer drugs, we performed CRISPR/Cas9 mediated mutagenesis of *ABCC1* and shRNA mediated knockdown of *ABCC1* in the human AML cell lines MOLM-13 and HL-60.

3.2.1. CRISPR/Cas9 mediated mutagenesis

Stable Cas9 expressing clones of the HL-60 (Fig. 6a) and MOLM-13 (Fig. 6b) cell lines were transduced with lentiviral vectors containing three different sgRNAs targeting *ABCC1* in exon 5 (sg*ABCC1.2*) and exon 8 (sg*ABCC1.1*, sg*ABCC1.3*). We also used two sgRNAs targeting the adeno-associated virus integration site 1 locus (*AAVS1*) as a negative control and two sgRNAs targeting the essential gene encoding the 60S ribosomal protein L17 (*RPL17*) as a positive control. The lentiviral plasmid expressing the sgRNAs also harbors a IRFP670 reporter, which allows easy monitoring of the proliferation dynamics between transduced

(sgRNA-expressing IRFP670 + cells) and untransduced (IRFP670 -) cells. If mutational disruption of a gene negatively affects cell proliferation, the levels of IRFP670 + cells will decrease over time. The depletion of cells transduced with sgRNAs targeting *RPL17* - an essential gene needed for protein synthesis - thus confirmed that the CRISPR/Cas9 system works in our cells. At the same time, levels of IRFP670-positive cells transduced with *AAVS1*-targeting sgRNAs remained stable over time, indicating that the cells can tolerate Cas9 induced double strand breaks in a locus that does not contain any genes. Cells transduced with sgRNAs targeting *ABCC1* did not deplete over time and thereby behaved similarly to the cells transduced with sgRNAs targeting *AAVS1*. This implies that *ABCC1* is not an essential gene and that CRISPR/Cas9-mediated mutagenesis of *ABCC1* does not interfere with cell proliferation under physiological conditions. The same pattern was observed in both HL-60 and MOLM-13 cells (Fig. 6).

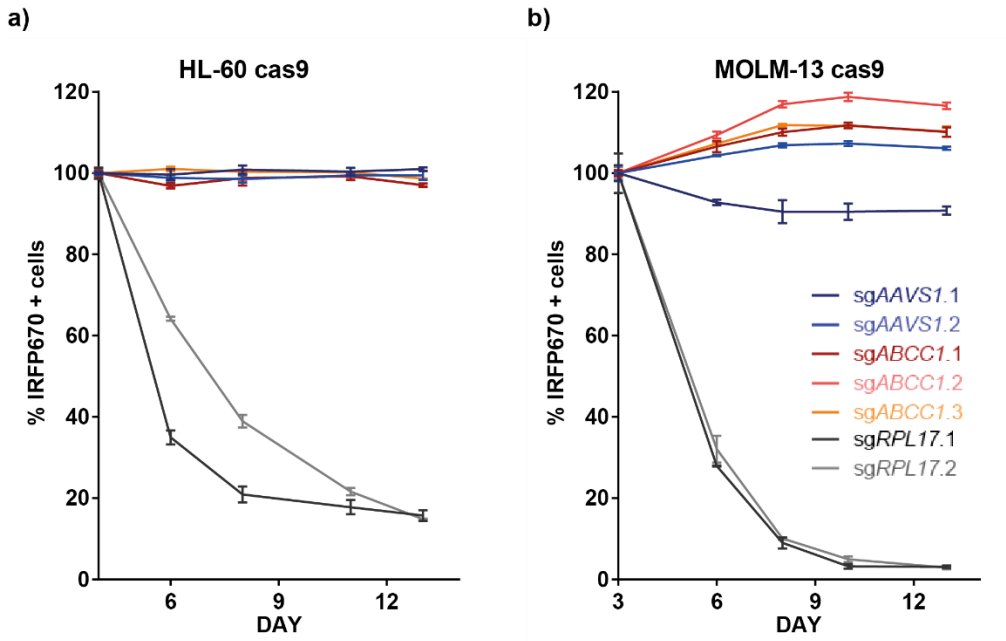


Figure 6: Effect of CRISPR/Cas9 mediated mutagenesis of *ABCC1* in Cas9-expressing (a) HL-60 and (b) MOLM-13 cells. Percentages of IRFP670+ cells transduced with indicated sgRNAs are depicted. Experiments were performed in triplicates and data was normalized to day 3 after transduction. Cells were split and measured every 2-3 days.

To assess the effect of CRISPR/Cas9-induced *ABCC1* loss on drug sensitivity, the mixed population of sgRNA-expressing vs untransduced cells was treated with Venetoclax and AZD-4320. In HL-60 cells, treatment with Venetoclax resulted in the depletion of cells expressing *ABCC1*-targeting sgRNAs, whereas the percentage of cells transduced with sgRNAs targeting

AAVS1 did not change over time after treatment (Fig. 7a). The same effect was observed when HL-60 and MOLM-13 cells were treated with AZD-4320 (Fig. 7b,c). This indicates that CRISPR/Cas9 mediated mutagenesis of *ABCC1* sensitizes AML cells to Venetoclax and AZD-4320.

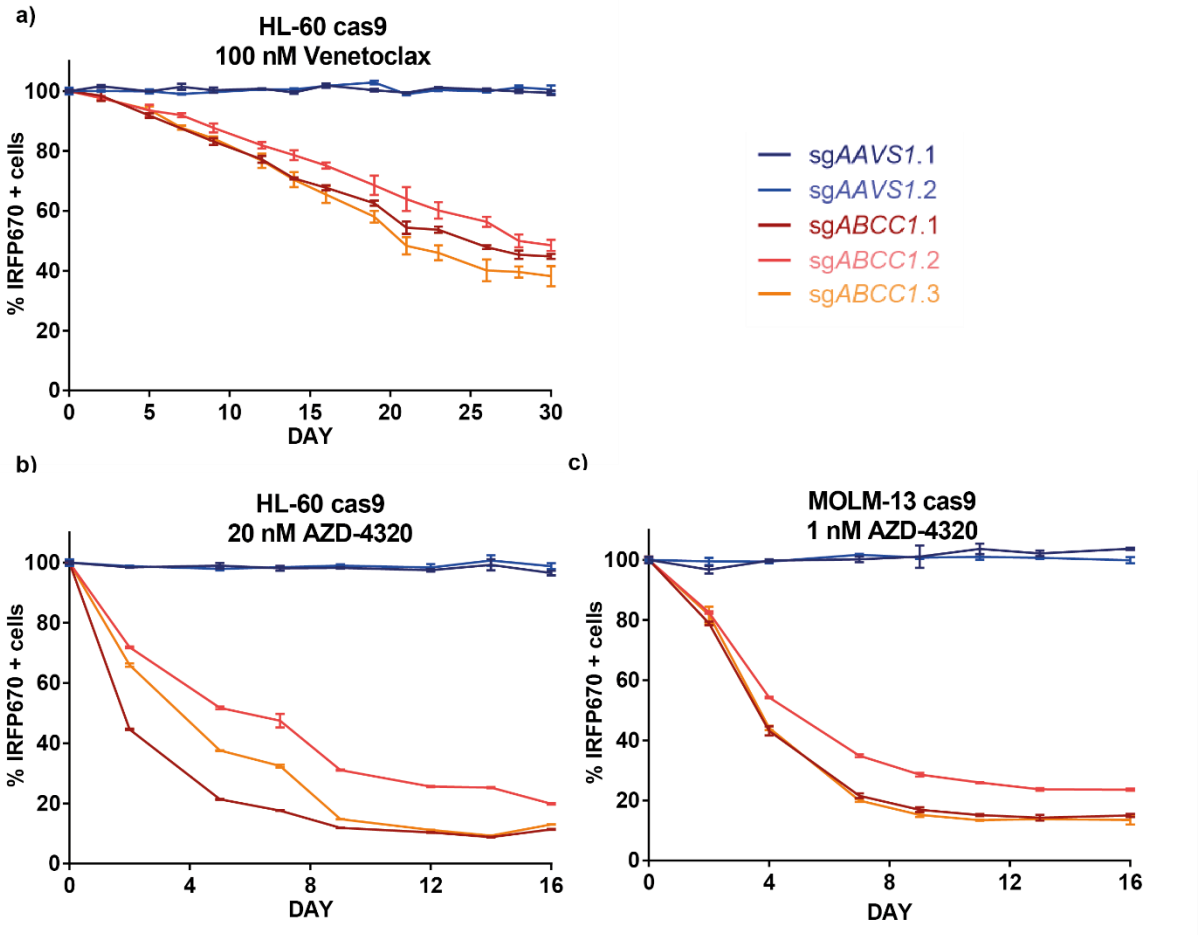


Figure 7: Proliferative competition assays of sg*ABCC1* expressing cells treated with BH3-mimetics. a) HL-60 cells treated with 100 nM Venetoclax. b) MOLM-13 cells treated with 1 nM AZD-4320. c) HL-60 cells treated with 20 nM AZD-4320. Percentage of IRFP670+ cells transduced with indicated sgRNAs is depicted, experiments were performed in triplicates and data was normalized to day 0 of drug treatment and to DMSO-treated control. Cells were split and measured every 2-3 days.

3.2.2. RNA interference (RNAi) of *ABCC1* in AML cell lines

The use of shRNAs allowed us to investigate the effect of *ABCC1* knockdown at the mRNA level. Another advantage of this strategy is that the lentiviral plasmid we used for shRNA expression contains a *Neomycin* resistance gene, which allowed us to select transduced cells to obtain homogenous populations of shRNA-expressing cells for gene expression analyses and viability assays. HL-60 and MOLM-13 cells were transduced with lentiviral vectors containing three different shRNAs targeting the *ABCC1* mRNA in the 3' untranslated region, two different shRNAs targeting *RPL17* as positive control for an essential gene and one shRNA targeting *Renilla* luciferase as a negative control. As *Renilla* luciferase is not expressed in human cells, this shRNA enabled us to evaluate whether the expression of shRNAs and subsequent activation of the RNAi pathway alone has an effect on cell viability. As expected, levels of cells transduced with the two RPL17-targeting shRNAs decreased strongly, while levels of cells expressing shRNAs targeting *Renilla* and *ABCC1* decreased mildly over time (data not shown). Knockdown efficiency was checked by RT-qPCR in fully selected cell populations. All three shRNAs targeting *ABCC1* induced a significant reduction of *ABCC1* mRNA levels compared to sh*Renilla*-expressing cells (Fig. 8a). Viability assays of the fully selected HL-60 cell populations showed that the expression of all three shRNAs targeting *ABCC1* lowered the IC_{50} values of Venetoclax, AZD-4320 and Etoposide (Fig. 8b). In the competition assay setup, cells transduced with *ABCC1*-targeting shRNAs depleted strongly when treated with AZD-4320 compared to DMSO (Fig. 8c,d). Taken together, these results show that knockdown of *ABCC1* sensitizes AML cells to Venetoclax and AZD-4320 treatment.

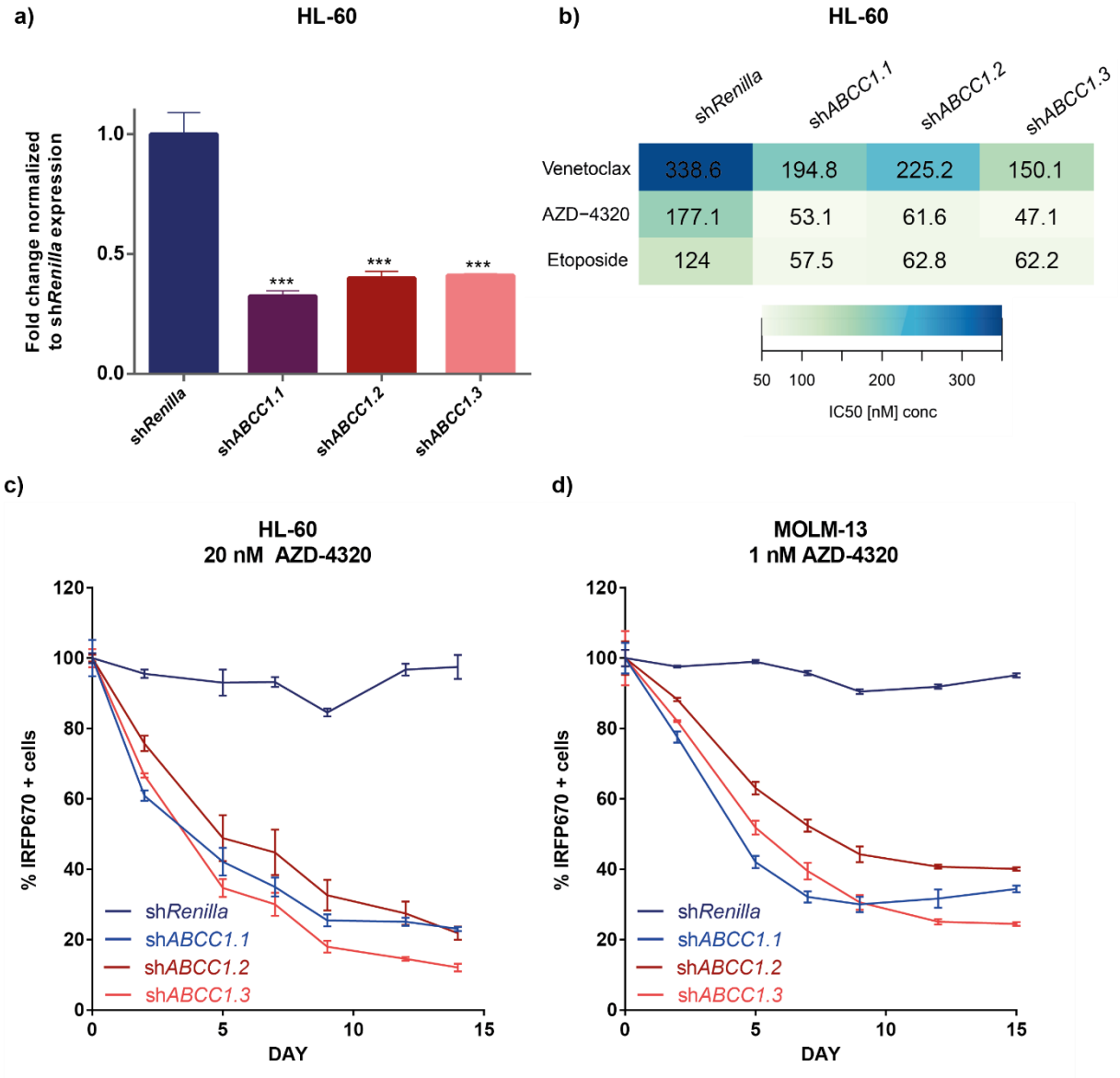


Figure 8: Effect of *ABCC1* knockdown. a) RT-qPCR analysis of *ABCC1* expression in fully selected HL-60 cells expressing indicated shRNAs, normalized to *ACTB* levels and expression levels of sh*Renilla* control. b) Heatmap of IC_{50} values [nM] of 5-day viability assays with fully selected HL-60 cells expressing indicated shRNAs (n=3). (c) Percentage of HL-60 cells transduced with indicated shRNAs treated with 20 nM AZD-4320 over time and normalized to day 0 of drug treatment and DMSO-treated control (n=3). d) Percentage of MOLM-13 cells transduced with indicated shRNAs treated with 1 nM AZD-4320 over time and normalized to day 0 of drug treatment and DMSO-treated control (n=3). c+d) Cells were split and measured every 2-3 days.

3.3.3. Generation and validation of *ABCC1* knockout clones

To validate the effect of *ABCC1* knockout in a homogenous population featuring a stable loss of *ABCC1*, we generated clonal populations of MOLM-13 cells with a homozygous *ABCC1* knockout. Single clones were obtained by serial dilution of the cell pools transduced with sgRNAs targeting *ABCC1*. The efficiency of Cas9-induced editing of the targeted locus was checked by using the tracking of INDELS by Decomposition (TIDE) tool (Fig. 9) (Brinkman et al. 2014). TIDE calculates the relative frequencies of Insertions/deletions (Indels) by aligning the sequence of targeted region to the untargeted control sequence. For this, the genomic regions targeted by the respective sgRNA are PCR amplified and analyzed by Sanger sequencing. By sequencing the pool of transduced cells, it is possible to check the efficiency of the genome editing process in the population and to determine which types of mutations predominate in the pool. For instance, while no editing of the *ABCC1*-locus was observed in sg*AAVS1*-expressing cells, almost 30% of the sequences in the pool of sg*ABCC1.1*-transduced MOLM-13 cells had a +1 insertion compared to the *wild type* reference (Fig. 9a). In addition, several other mutational events were present at lower frequencies and about 5% of sequences were not targeted (Fig. 9b). By analyzing a clone with TIDE, the exact type of Indels on individual alleles can be identified. For instance, sequences from the *ABCC1* locus of a clone derived from cells transduced with sg*ABCC1.1* harbored a +1 and a +3 insertion relative to the predicted position of the Cas9-induced double strand break at a 1:1 ratio representative of two alleles (Fig. 9c). Clones with mutations that most likely lead to loss or reduction of ABCC1 were used for further validation.

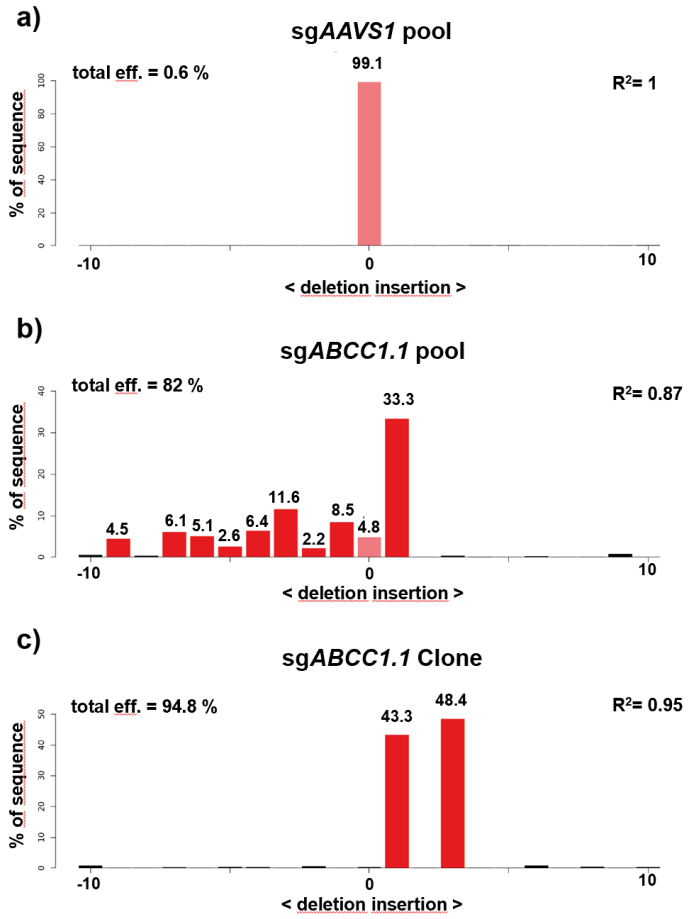


Figure 9: Tracking of Indels by Decomposition analysis. a) MOLM-13 Cas9 cells transduced with sg*AAVS1.1* served as a control for untargeted *ABCC1*. b) Pooled population of MOLM-13 Cas9 cells transduced with sg*ABCC1.1* pool. c) sg*ABCC1.1* MOLM-13 Cas9 clone. a-c) The bar charts t show the spectrum of indels and their frequencies.

For the identification of the exact mutational changes on both alleles, the same PCR product used for the TIDE analysis was cloned into a linearized vector by TA cloning. Sanger sequencing of the inserts identified that *ABCC1* knockout clone 1 harbored a -3 deletion of nucleotides on position 94962-94964 of the *ABCC1* gene and at the same time a +6 insertion of the bases *AACCCC* on the same position (NM004996:g.94962-94964delins*AACCCC*). This results in an exchange of Leucine to Asparagine and Proline at position 313 (p.Leu313delinsAsnPro) on one allele. On the other allele there was a +1 insertion of thymine between nucleotides 94963-94964 in the *ABCC1* gene (NM004996:g.94963-94964ins*T*), resulting in a frame shift leading to an exchange of Phenylalanine to Valine on position 314 followed by a *STOP* codon (Fig. 10a). In *ABCC1* knockout clone 2 we identified a +1 insertion

of thymine between nucleotides 66984-66985 in the *ABCC1* gene on one allele (NM004996:g.66984-66985ins*T*). This resulted in a frameshift inducing an amino acid change of Serine to Leucine at position 199 followed by a *STOP codon* at the 8th amino acid after the first change. On the second allele we found a deletion of bases 66982-66990 and an insertion of *AAGTCAC* at the same position in the *ABCC1* gene (NM004996:g.66982_66990delins*AAGTCAC*). This mutation led to the exchange of Phenylalanine198 to a *STOP* codon (p.Phe198*) (Fig. 10b). In summary, both clones that were characterized in detail harbored mutations in the *ABCC1* gene – including at least one nonsense mutation leading to a dysfunctional protein. Further functional investigation using Etoposide – a known substrate of ABCC1 - showed that both clones were more sensitive to Etoposide compared to the sg*AAVS1.1* clone, in which the *ABCC1* gene is intact (data not shown). Based on these observations, we chose to continue to use these two clones for subsequent experiments. In addition to the increased sensitivity to Etoposide, both *ABCC1*-mutated clones were more sensitive to Venetoclax and AZD-4320 compared to the *AAVS1* clone (Fig 10c). No difference in Midostaurin sensitivity was observed between *ABCC1* knockout clones and the *AAVS1* clone (Fig. 10c). This confirms that mutational inactivation of ABCC1 sensitizes AML cells to BCL-2 inhibitors, but not to other targeted drugs.

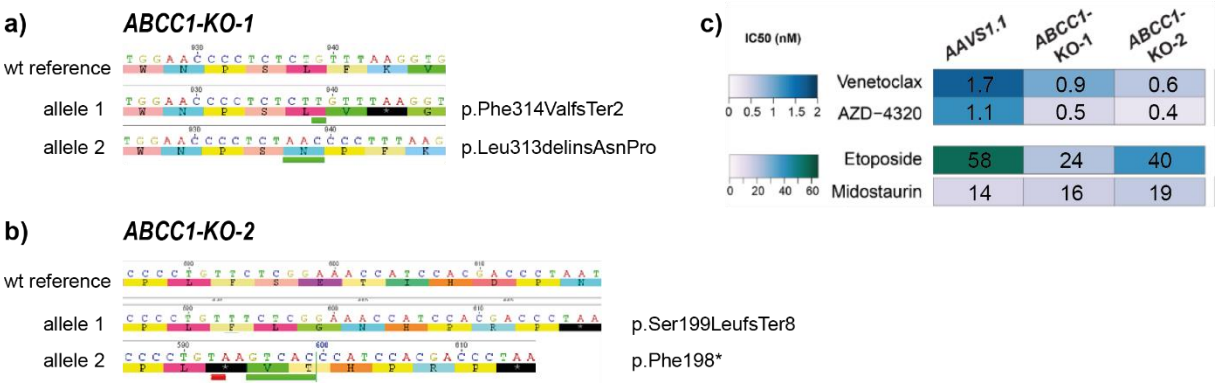


Figure 10: Characterization of MOLM-13 *ABCC1* knockout clones. a) Sequence changes on both alleles of *ABCC1-KO-1* clone b) Sequence changes on both alleles of *ABCC1-KO-2* clone c) Heatmap of IC_{50} values [nM] of 5-day viability assays with Venetoclax, AZD-4320, Etoposide and Midostaurin in MOLM-13 clones (*AAVS1* and *ABCC1-KO-1/ -2*, n=3).

As viability assays only reflect drug effects in a timeframe of a few days, we next aimed to investigate the long term effects of ABCC1 loss in combination with different drug treatments. When treated with DMSO, all three clones proliferated at the same growth rate, irrespective of *ABCC1* genotype (Fig. 11). However, when treated with Venetoclax, both *ABCC1* knockout

clones grew at a slower rate compared to the *AAVS1* clone (Fig. 11a). *ABCC1* knockout clones were unable to sustain treatment with 1 nM AZD-4320 for more than a week, while the *AAVS1* clone proliferated exponentially under these conditions (Fig. 11b). Treatment with increasing concentrations of the MCL-1 specific inhibitor AZD-5991, which is structurally distinct from the BCL-2 inhibitors Venetoclax and AZD-4320, had little effect on cell growth of the *ABCC1* knockout clones (Fig. 11c). These data show that *ABCC1* knockout cells have a proliferative disadvantage when treated with Venetoclax and AZD-4320, which is in line with prior experiments in pooled cell populations.

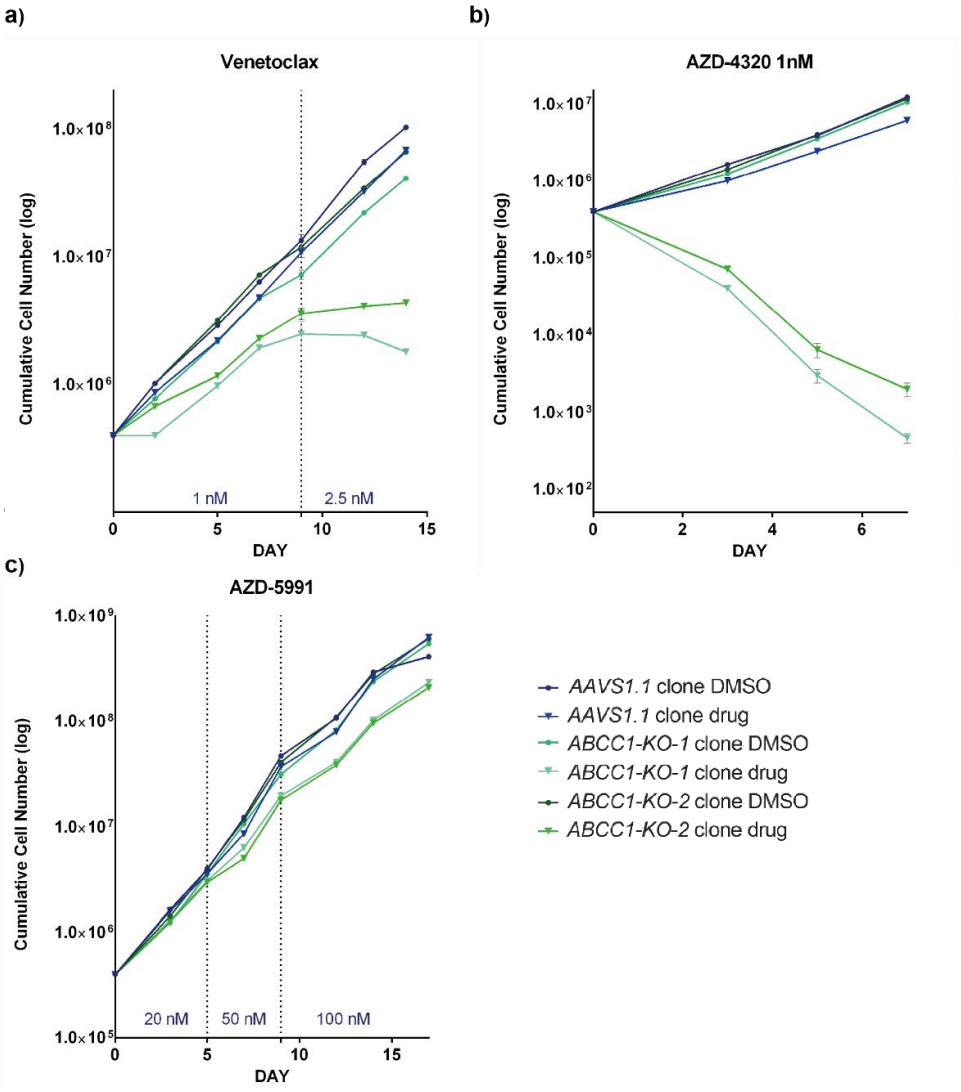
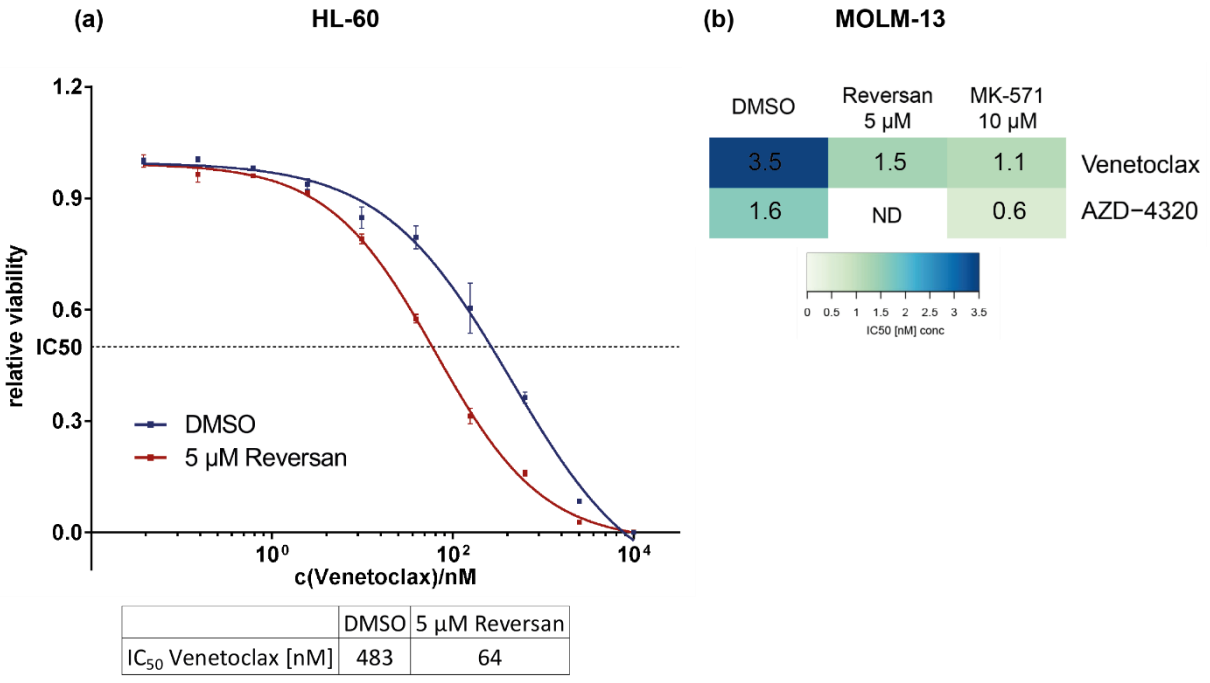


Figure 11: Growth curves of MOLM-13 *ABCC1* knockout clones. a) Cells were treated with increasing Venetoclax concentrations (b) 1 nM AZD-4320 or (c) increasing AZD-5991 concentrations. Experiments were performed in duplicates (AZD-4329, AZD-5991) or triplicates (Venetoclax). Cells were split and measured every 2-3 days.

3.3. Pharmacological inhibition of ABCC1

To further evaluate the effect of ABCC1 inhibition on drug sensitivity, we exposed cell lines to combinatorial treatment with ABCC1 inhibitors and Venetoclax or AZD-4320. AML cell lines were not sensitive to the ABCC1 inhibitors Reversan and MK-571 (Table 3). However, treatment with 5 µM Reversan drastically sensitized HL-60 cells to Venetoclax, resulting in a 7.5-fold drop of the IC_{50} from 483 nM (DMSO control) to 64 nM (Reversan treatment, Fig. 12a). The same effect was observed in MOLM-13 cells, where co-treatment with 5 µM Reversan sensitized the cells to Venetoclax (Fig. 12b). Co-treatment with MK-571 – another ABCC1 inhibitor – also sensitized MOLM-13 cells to both Venetoclax and AZD-4320 (Fig. 12b). Thus, these data confirm that besides the complete loss or reduction of ABCC1 protein, pharmacologic inhibition of ABCC1 transporter function is sufficient to sensitize AML cells to Venetoclax and AZD-4320 treatment.



	DMSO	5 µM Reversan
IC_{50} Venetoclax [nM]	483	64

Figure 12: Combinatorial treatment of ABCC1 inhibitors with BH3-mimetics. a) 5-day dose-response curve of Venetoclax in HL-60 cells co-treated with either DMSO or 5 µM Reversan (n=3). b) Heatmap of IC_{50} values [nM] of 5-day viability assays with Venetoclax and AZD-4320 in MOLM-13 cells co-treated with either DMSO, 5 µM Reversan or 10 µM MK-571 (n=3).

3.4. Generation, characterization and re-sensitization of Venetoclax-resistant MOLM-13 Cas9 cells

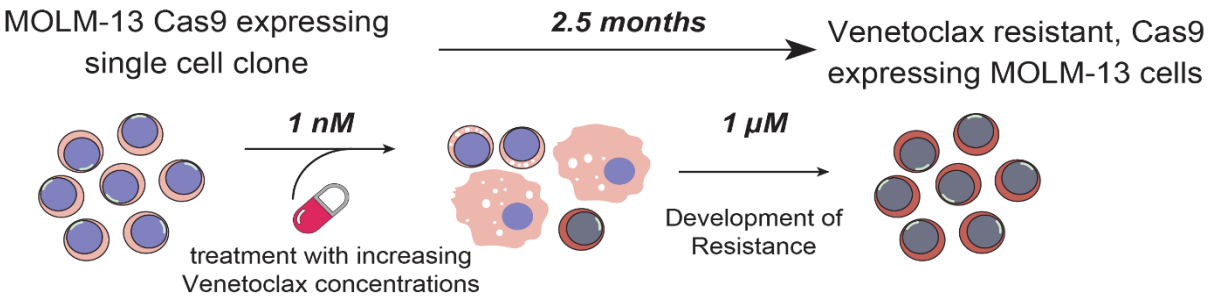


Figure 13: Schematic representation of the generation of Venetoclax-resistant Cas9 expressing MOLM-13 cells. Cells were treated for 2.5 months with increasing concentrations of Venetoclax, until they grew in media supplemented with 1 µM Venetoclax.

In order to discover potential Venetoclax resistance mechanisms and to apply possible re-sensitization strategies, we generated a Venetoclax-resistant cell line (Fig. 13). Cas9 expressing MOLM-13 cells were treated with increasing Venetoclax concentrations for up to 2.5 months until they exhibited stable growth kinetics in medium supplemented with 1 µM Venetoclax, which is 1000 times higher than the concentration used at the start of the experiment. RT-qPCR revealed that the Venetoclax-resistant MOLM-13 cells expressed *BCL-2*, *BCL-X*$_L$ and *MCL-1* at higher levels than the parental cell line (Fig. 14a). No significant change in the amount of *ABCC1* mRNA was observed between resistant and parental cells (Fig. 14a). CRISPR/Cas9 mediated mutagenesis of *ABCC1* lead to a depletion of Venetoclax-resistant MOLM-13 cells when cultured in 1 µM Venetoclax compared to sgAAVS1-expressing cells (Fig. 14b). This indicates that loss of *ABCC1* can re-sensitize resistant AML cells to Venetoclax.

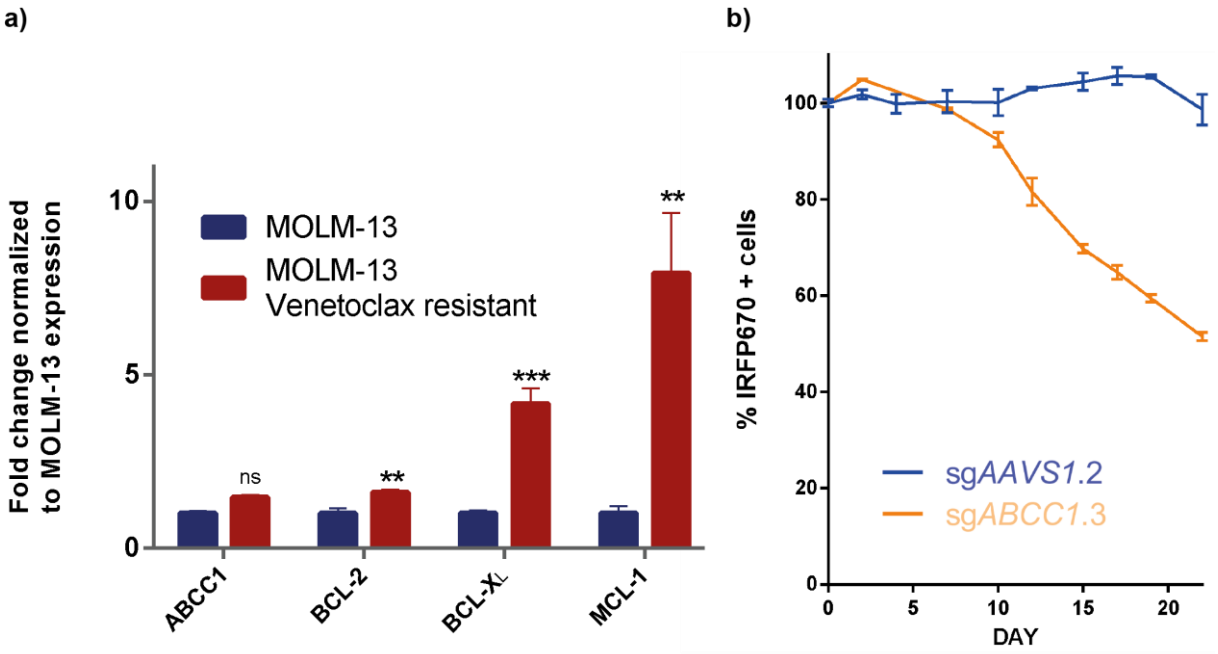


Figure 14: Characterization and re-sensitization of Venetoclax resistant MOLM-13-Cas9 cells. a) RT-qPCR analysis of mRNA expression of *ABCC1*, *BCL-2*, *BCL-X*$_L$ and *MCL-1* normalized to *ACTB* levels and the parental cell line (n=3). Competition assays of re-sensitization of MOLM-13-Cas9 Venetoclax resistant cells by CRISPR/Cas9-induced mutational inactivation of *ABCC1.* Cells cultured in 1 µM Venetoclax. Percentages of IRFP670+ cells transduced with indicated sgRNAs were normalized to day 0 of drug treatment and to DMSO-treated control (n=3).

3.5. Investigation of the role of ABCC1 in primary patient-derived AML cells

Even though cell lines are a powerful tool to investigate a variety of biological processes, findings obtained in cell lines do not always represent what is occurring in patients, where many additional factors can influence disease development, progression and treatment response. For this reason, we used primary patient-derived AML cells to investigate the role of ABCC1 in the response to BCL-2 inhibitors.

3.5.1. The influence of ABC transporter expression on Venetoclax treatment response

To determine which ABC transporters are expressed in AML cells and whether high expression of ABCC1 correlates with poor response to Venetoclax treatment, we analyzed the expression of multiple relevant ABC transporters patient derived AML cells by RT-qPCR. All patients in this cohort (n=16) were treated with Venetoclax and the samples were taken shortly before

Venetoclax treatment was initiated. We found that of the three most prominently studied ABC transporters in MDR, *ABCC1* was expressed at highest levels. While *ABCB1* was also expressed in the majority of samples at intermediate levels, *ABCG2* expression could not be detected (Fig. 15). In addition, *ABCC1* was the highest expressed ABC transporter among the members of the MRP subfamily we examined. While *ABCC3*, *ABCC4*, *ABCC5* and *ABCC10* were consistently expressed at lower levels, no expression of *ABCC2* and *ABCC6* was detected. Thus, ABCC1 is highly expressed in AML cells, suggesting that it plays an important role in AML cells.

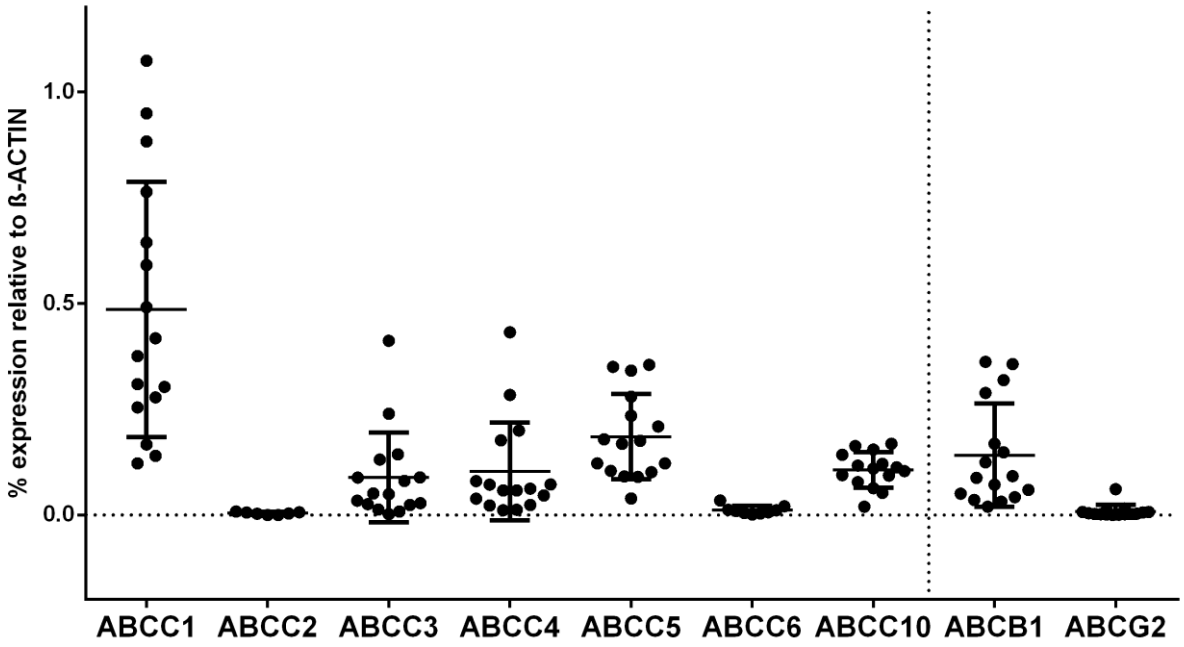


Figure 15: Expression of ABC Transporters in AML patient samples. n=16, % expression relative to *ACTB* = 100*2^(-Δc(t)(*ß-ACTIN*)), experiments were performed in duplicates.

To evaluate the potential use of ABC transporter expression as prognostic marker for the response to Venetoclax treatment, we compared the expression of different ABC transporters between good and poor responders to Venetoclax treatment, according to information obtained from our clinical collaboration partners. For this analysis, we excluded *ABCC2* and *ABCC6*, as these transporters were not expressed in the examined samples and divided the patients into two categories: Good responders had a complete remission (CR) (n=2) or a complete

remission with incomplete hematologic recovery (Cri) (n=2) after Venetoclax treatment, while poor responders reached cytoreduction (n=7) or stable disease (n=3) after Venetoclax treatment. Patients who did not respond at all to Venetoclax treatment (non responders) were excluded from this analysis (n=2). This analysis showed that patients with a poor response to Venetoclax treatment had higher significantly higher levels of *ABCC1* expression compared to good responders (Fig. 16). The expression levels of other ABC transporters we investigated was not different between poor and good responders. In summary, the RT-qPCR of patient-derived primary AML cells identified *ABCC1* as the highest expressed ABC transporter of all examined ones in this cohort. Furthermore, our data indicate that high expression of *ABCC1* could be associated to a poor response to Venetoclax treatment.

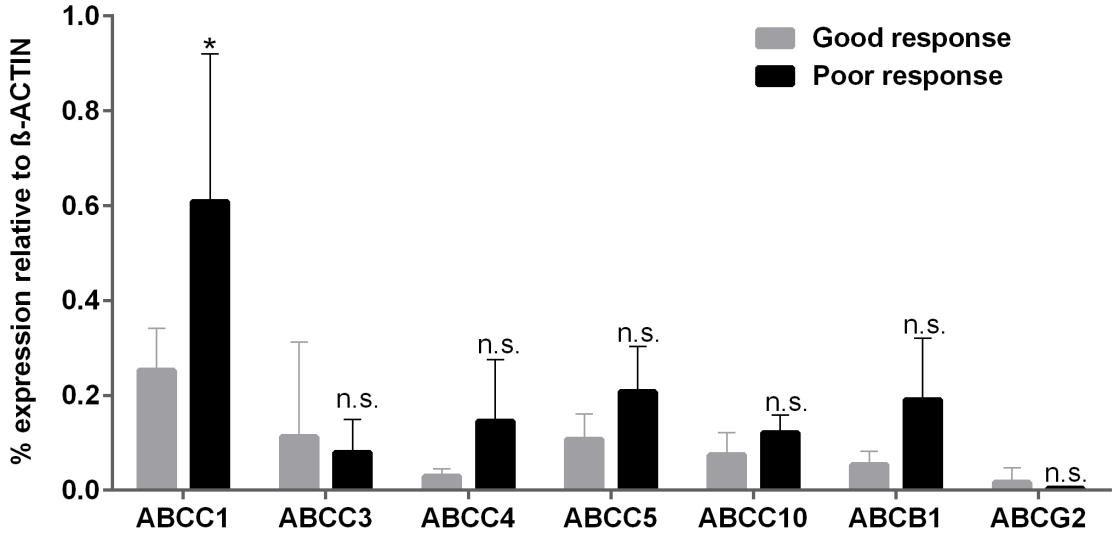


Figure 16: Comparison of expression of ABC transporters between patients with good or poor response prior to start with Venetoclax treatment. Patients with good response (n=4) consisted of patients with complete remission (CR) (n=2) or complete remission with incomplete hematologic recovery (Cri) (n=2). patients with a poor response (n=10) were defined with patients having cytoreduction (n=7) or a stable disease (n=3). Values shown are the mean expression of the respective patient cohort, and experiments were performed in duplicates.

3.5.2. Co-treatment of primary patient derived AML cells with Reversan and BCL-2 inhibitors

To obtain additional insight into the relevance of our findings we treated primary patient-derived AML cells with Venetoclax and AZD-4320 in combination with Reversan to generate dose response curves and determine differences in viability between the different treatments.

Table 4: IC_{50} values [nM] of Venetoclax in combination with 2.5 µM or 5 µM Reversan of AML patient samples

	DMSO	2.5 µM Reversan	5 µM Reversan
P1 (***DNMT3A***, ***CEBPA***, ***EZH2***, ***U2AF1***)	28.5	n.d.	9.5
P2 (***FLT3*** **ITD pos.**)	>10000	n.d.	>10000
P3	>5000	>5000	>5000
P4 (***FLT3*** **ITD pos.**, ***TET2***)	>20000	>20000	>20000
P5 (***DNMT3A***, ***WT1***)	154.9	79.0	70.5

Table 5: IC_{50} values [nM] of AZD-4320 in combination with 2.5 µM or 5 µM Reversan of AML patient samples

	DMSO	2.5 µM Reversan	5 µM Reversan
P1 (***DNMT3A***, ***CEBPA***, ***EZH2***, ***U2AF1***)	25	n.d.	1.4
P2 (***FLT3*** **ITD pos.**)	440.5	n.d.	20.8
P3	111.7	13.9	6.7
P4 (***FLT3*** **ITD pos.**, ***TET2***)	1259	630.9	445.4
P5 (***DNMT3A***, ***WT1***)	39.3	8.4	4.8

The IC_{50} values obtained from dose-response curves of five primary patient-derived AML patient samples showed a very heterogeneous response to Venetoclax and AZD-4320 (Table 3 and Table 4). In general, AZD-4320 was more potent than Venetoclax, resulting in lower IC_{50} values. Cells from patients 2, 3 and 4 were the least sensitive to Venetoclax treatment. In comparison to the tested cell lines (Table 3), primary patient samples where more sensitive to Reversan treatment alone (Fig. 17). Combinatorial treatment with Reversan sensitized patient-derived AML cells to Venetoclax and AZD-4320 treatment. With increasing Reversan

concentrations, AML cells became even more sensitive to both compounds. For most samples (especially P1, P2, P3) the antiproliferative effects of combined Reversan and AZD-4320 treatment was stronger than the sum of the inhibitory effects of both drugs alone (Fig. 17a). For instance, co-treatment with 2.5 µM Reversan rendered patient sample 3 almost 10 times more sensitive to AZD-4320 treatment (Table 5, Fig. 17a). As higher doses of Venetoclax than AZD-4320 were required to inhibit the growth of AML cells, Reversan only moderately sensitized them to Venetoclax (Fig. 17b). However, the increase in sensitivity to BCL-2 inhibitors is still in line with our results obtained in cell lines that were co-treated with Reversan. Therefore, these data confirm the relevance of ABCC1 as an important mediator of drug response in patients.

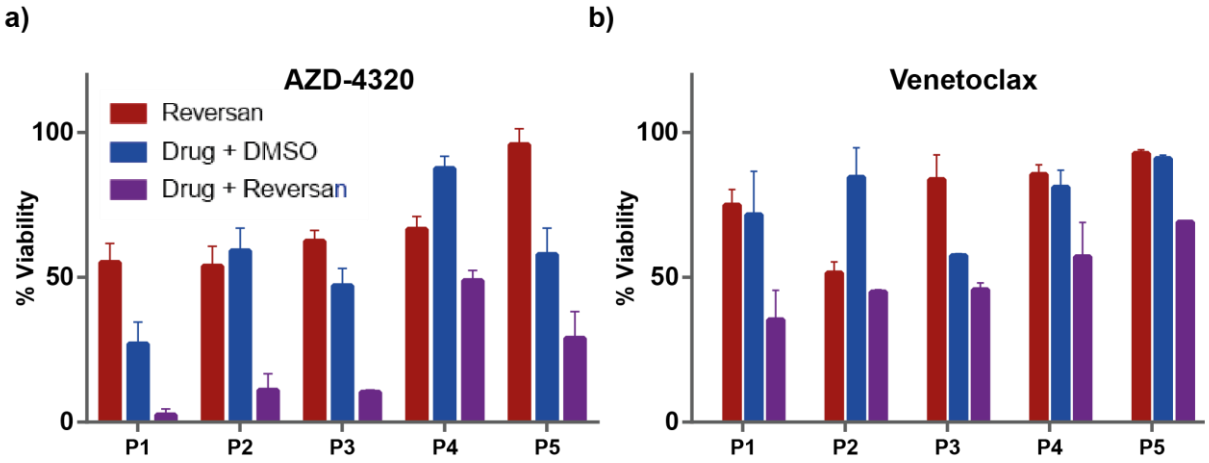


Figure 17: Relative viability of primary patient-derived AML cells treated with Reversan in combination with BCL-2 inhibitors at selected concentrations. AZD-4320 (a) and Venetoclax-treated samples (b) were treated for 3 days and viability was assessed with CellTiter-Glo® (Promega) (n=3). Data is normalized to DMSO treated control. Concentrations for AZD-4320 treated samples (a): P1 (5 µM Reversan, 156 nM AZD-4320), P2 (5µM Reversan, 156 nM AZD-4320), P3 (2.5 µM Reversan, 156 nM AZD-4320), P4 (2.5 µM Reversan, 39 nM AZD-4320), P5 (2.5 µM Reversan, 39 nM AZD-4320).; Concentrations for Venetoclax treated samples (b): P1 (5 µM Reversan, 39 nM Venetoclax), P2 (5µM Reversan, 39 nM Venetoclax), P3 (2.5 µM Reversan, 390 nM Venetoclax), P4 (2.5 µM Reversan, 1.25 µM Venetoclax), P5 (2.5 µM Reversan, 4.8 nM Venetoclax).

3.6. The role of glutathione (GSH) in the response to BCL-2 inhibition

Because ABCC1 – in contrast to most other ABC transporters (Gauthier et al. 2013) – often exports drugs together with GSH and is able to transport GSH-conjugates (Cole and Deeley 2006), we aimed to inhibit Glutathione metabolism with different compounds in order to investigate the role of GSH in the response to BCL-2 inhibitors.

3.6.1. The effect of inhibition of glutathione (GSH) synthesis on the sensitivity to BH3-mimetics

As glutathione (GSH) is an important co-factor of ABCC1-mediated drug efflux, we investigated the role of GSH in modulating BCL-2-inhibiton by perturbation of GSH synthesis using Buthionine Sulfoximine (BSO). We monitored cell growth of Venetoclax- or AZD-4320-treated MOLM-13 cells in combination with 100 µM BSO over 2-3 weeks. AML cell lines were not sensitive to BSO in general (Table 3) and the proliferation of MOLM-13 cells was only slightly reduced when treated with 100 µM BSO compared to the DMSO control. However, when BSO and Venetoclax were combined, cell proliferation was significantly reduced as compared to treatment with Venetoclax or BSO alone. The combinatorial treatment of BSO with increasing Venetoclax concentrations induced a growth arrest in MOLM-13 cells (Fig. 18a). The same effect was observed in combinatorial treatment of AZD-4320 and BSO (Fig. 18b). This data strongly indicates that GSH is important for the maintenance of AML cell sensitivity to Venetoclax and AZD-4320.

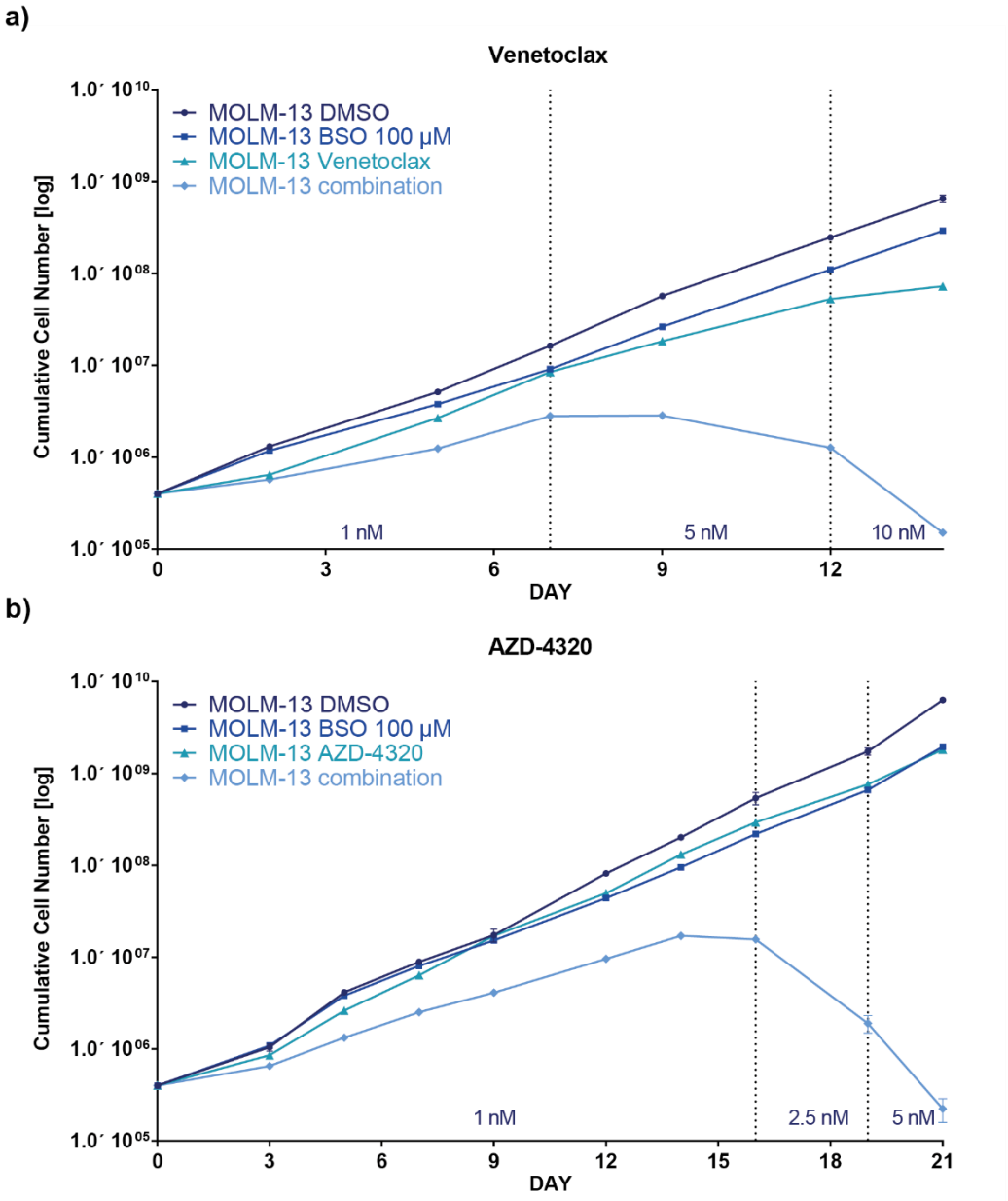
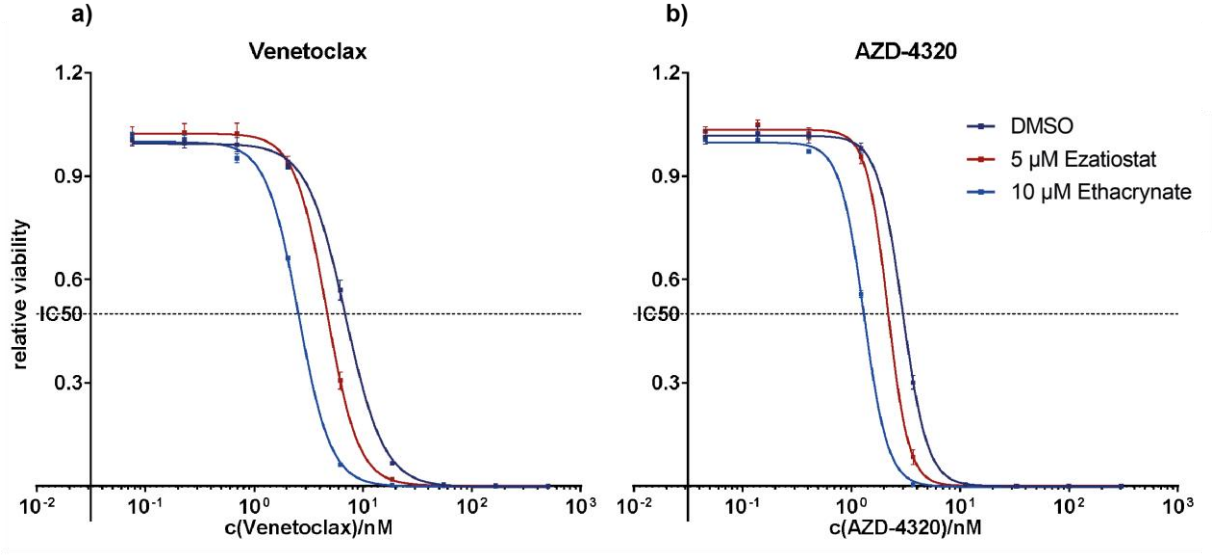


Figure 18: Effect of inhibition of GSH synthesis combined with BH3-mimetics on cell proliferation. a) Growth curves of MOLM-13 cells treated with DMSO, 100 µM BSO, increasing Venetoclax concentrations and increasing Venetoclax concentrations combined with 100 µM BSO. b) Growth curves of MOLM-13 cells treated with DMSO, 100 µM BSO, increasing AZD-4320 concentrations and AZD-4320 concentrations combined with 100 µM BSO. Experiments were performed in duplicates and cells were split and measured every 2-3 days.

3.6.2 Inhibition of Glutathione-S-Transferase (GST) combined with BCL-2 Inhibition

Given the fact that there are multiple ways how GSH can influence the cellular response to drugs, we wanted to further elucidate the mechanism behind the sensitization of AML cells to Venetoclax and AZD-4320 treatment that was observed upon inhibition of GSH metabolism. We performed combinatorial treatments of the GST inhibitors Ethacrynic acid and Ezatiostat with Venetoclax and AZD-4320 to investigate whether inhibition of glutathione transfer to target substances affects the effect of Venetoclax and AZD-4320. Similar to BSO treatment, treatment with ethacrynic acid or Ezatiostat alone had little effect on AML cell survival (Table 3). However, addition of 10 µM Ethacrynic acid or 5 µM Ezatiostat sensitized MOLM-13 cells to AZD-4320 and Venetoclax treatment in 5 day viability assays, with Ethacrynic acid showing the stronger effect (Fig. 19a,b).



	DMSO	5 µM Ezatiostat	10 µM Ethacrynate
IC_{50} Venetoclax [nM]	6.887	4.626	2.555

	DMSO	5 µM Ezatiostat	10 µM Ethacrynate
IC_{50} AZD-4320 [nM]	2.955	2.156	1.306

Figure 19: Effect of GST inhibition on sensitivity of MOLM-13 cells to BH3-mimetics. a) 5-day dose response curve of AZD-4320 in MOLM-13 cells in combination with either DMSO, 10 µM Ethacrynate or 5 µM Ezatiostat. b) 5-day dose response curve of Venetoclax in MOLM-13 cells in combination with either DMSO, 10 µM Ethacrynate or 5 µM Ezatiostat. n=3.

4. Discussion

ABCC1 is an important player in multi-drug resistance in cancer and xenobiotic substrates transported by ABCC1 include a variety of cytostatic drugs like anthracyclines, alkaloids and podophyllotoxins (Cole 2014b). However, so far it is not known whether recently approved targeted therapies are also substrates of ABCC1. Knowledge about the interactions between drugs and transporter proteins is important, as co-administration of drugs could influence transporter activity. Resulting changes in pharmacokinetics might reduce drug efficacy or increase their toxicity. Therefore, the aim of this work was to investigate the influence of genetic and pharmacological inhibition of ABCC1 on the efficacy of newly approved targeted drugs – especially BH3-mimetics – in AML cells. Furthermore, we investigated the role of GSH and GSTs in modulating the sensitivity to BH3-mimetics. Finally, we aimed to confirm the relevance of our findings in patient derived AML cells by treating them with BH3-mimetics in combination with the ABCC1 inhibitor Reversan and analyzing the expression levels of ABCC1 among other relevant ABC transporters in patient derived AML cells.

In this study, we mainly used the HL-60 [*MYC* amplification] and MOLM-13 [*FLT3*-ITD; *MLL-AF9*] cell lines, because they showed intermediate (HL-60) and high sensitivity (MOLM-13) to the majority of the compounds we used (Table 3). It would have been difficult to perform mechanistic studies in cell lines that are intrinsically resistant to the tested compounds, such as the THP-1 cell line, which harbors a *TP53* mutation, which is a known resistance mechanism to BCL-2 inhibition (Nechiporuk et al. 2019).

Perturbation of *ABCC1* by CRISPR/Cas9-mediated mutagenesis and by shRNA-mediated knockdown alone had no negative effect on cell growth in all cell lines we tested, which is in line with previous findings indicating that *ABCC1* is not an essential gene (Wijnholds et al. 1997). However, it was observed that *ABCC1* knockout cells harbor increased levels of intracellular GSH and show increased sensitivity to chemotherapeutic drugs like Etoposide (Lorico et al. 1997). By showing Etoposide hypersensitivity, we confirmed the effect of *ABCC1* knockout and *ABCC1* knockdown in addition to genotyping and RT-qPCR in AML cell lines. For future experiments, Western blot or measurements of ABCC1 protein levels by intracellular flow cytometry could provide additional proof of the loss or reduction of the protein in our cellular models.

Even though loss or reduction of ABCC1 had no effect on cell growth, cells with impaired ABCC1 exhibited increased sensitivity to Venetoclax and AZD-4320 treatment. In the

competition assay setup, *ABCC1* knockout cells depleted more strongly when treated with AZD-4320 compared to Venetoclax. This could either be due to intrinsically higher sensitivity to this compound, which could be reflected by a lower IC_{50} value for this drug in the parental cell lines compared to Venetoclax, or due to the effect of combined BCL-2 and BCL-X_L inhibition by AZD-4320. Alternatively, slightly different biophysical properties, such as its higher hydrophobicity (Patterson et al. 2021) could make AZD-4320 a more specific substrate of ABCC1. To rule out that inhibition of ABCC1 sensitizes cells to anticancer drugs in general, we treated *ABCC1* knockout clones with Midostaurin and AZD-5991 (Fig. 10b and Fig. 11c). No significant differences in sensitivity between *ABCC1* wildtype and *ABCC1* knockout cells were observed with those two targeted therapies.

Similar to genetic inhibition of *ABCC1*, monotherapy treatment with the ABCC1 inhibitors Reversan and MK-571 had neglectable effects on cell survival (Table 3). However, incubation with ABCC1 inhibitors significantly lowered the IC_{50} values of Venetoclax and AZD-4320 in HL-60 and MOLM-13 cells. Even though MK-571 and Reversan are often used to assess the effect of ABCC1 transporter function on drug response, off-target effects of these compounds in addition to the inhibition of ABCC1 could also influence the cellular response to BCL-2 inhibition. both MK-571 and Reversan were identified decades ago and have not been optimized further for targeted ABCC1 inhibition. Nevertheless, as both compounds confirmed the effects we observed with genetic inhibition of *ABCC1*, we are confident that drug-induced impairment of ABCC1 function indeed causes cells to be more sensitive to BCL-2 inhibition.

A striking issue we encountered with the MOLM-13 cell line was the rapid development of resistance to Venetoclax after prolonged exposure. To counteract to this effect, we gradually increased Venetoclax concentrations in long term experiments. Because the adaption to Venetoclax was apparent within few weeks of exposure, we hypothesized that this is due to a change in apoptotic priming (Bhatt et al. 2020) rather than clonal outgrowth of cells harboring specific mutations leading to Venetoclax resistance, like the Gly101Val mutation in BCL-2 (Thangavadivel and Byrd 2019), loss of *BAX* or *TP53* (Nechiporuk et al. 2019) or *OPA1* overexpression (Chen et al. 2019). Indeed, RT-qPCR analysis showed increased expression of *MCL-1* and *BCL-X$_L$* in Venetoclax-resistant MOLM-13 cells, suggesting that the Venetoclax-resistant MOLM-13 cells rely more on MCL-1 and BCL-X_L for keeping the balance of pro- vs. anti-apoptotic factors in favor of cell survival than on BCL-2. Although the Venetoclax-resistant MOLM-13 cells did not show a significant increase in *ABCC1* expression, we observed that cell lines that overexpress *ABCC1* are more resistant to Venetoclax (data not shown). The fact

that the Venetoclax-resistant MOLM-13 could be re-sensitized by *ABCC1* knock-out is additional evidence for the importance of ABCC1 in modulating the response of AML cells to Venetoclax.

The synergistic effect between BSO treatment and BCL-2 inhibition suggests that GSH has an essential role in the response of AML cells to Venetoclax and AZD-4320. BSO was successfully used in the past to re-sensitize ABCC1-overexpressing cells to ABCC1 substrates Etoposide and Daunorubicin (Benderra et al. 2000). Our results indicate that Venetoclax and AZD-4320 might be effluxed by ABCC1 in combination with GSH. This would also explain the specificity of the synergy between BCL-2 inhibitor treatment and perturbation of ABCC1, but not other ABC transporters, as ABCC1 is one of the only ABC transporters that effluxes substrates together with GSH. To verify that the synergistic effect of BSO treatment and BCL-2 inhibition is caused by depletion of intracellular glutathione levels, it would be interesting to measure intracellular GSH levels after BSO treatment. Another strategy to deplete cells of GSH would be by knocking out the Cystine/glutamate antiporter xCT (*SLC7A11*), or by treating cells with SLC7A11 inhibitors (e.g. Sulfasalazine). SLC7A11 imports extracellular cystine which is converted to cysteine, the rate-limiting precursor for GSH biosynthesis (Fig. 3) (Koppula et al. 2018). As blockage of SLC7A11 leads to GSH depletion (Koppula et al. 2018), Sulfasalazine treatment would be expected to synergize with BCL-2 inhibition. This would further strengthen our assumption that GSH has a key role in modulating the efficacy of BCL-2 inhibitors.

Additional evidence for the importance of GSH in the response to BCL-2 inhibition was the sensitization to Venetoclax and AZD-4320 by combinatorial treatment with GST inhibitors. GSTs could therefore play a role in the detoxification of Venetoclax and AZD-4320 by conjugating GSH to these compounds. The compounds we used target GSTs by two different mechanisms: Ethacrynic acid binds to the hydrophobic H-Site of GSTs, while Ezatiostat is a glutathione peptidomimetic (Singh and Reindl 2021). The stronger effect of Ethacrynic acid compared to Ezatiostat could be explained by the different target mechanism of GST inhibitors, different concentrations used or by different off-target effects. Ethacrynic acid is used as a loop diuretic and its GST-inhibiting properties were discovered later, while Ezatiostat was specifically developed as an GST inhibitor (Zhang et al. 2021). GSTs are also known to protect cells from apoptosis by inhibiting c-Jun N-terminal kinase (JNK) (Allocati et al. 2018). Inhibition of GSTs results in the phosphorylation of c-Jun by JNK, leading to subsequent activation of AP-1 target genes, which could be an alternative explanation for the increased potency of BCL-

2 inhibitors when combined with GST inhibitors. Given the fact that GSTs have so many different roles in cell physiology apart from drug metabolism, a more detailed characterization of their role in response to BCL-2 inhibition would have been beyond the scope of this work. The use of GST inhibitors was another tool for us to validate the role of GSH in the response to Venetoclax and AZD-4320 treatment. However, to further clarify the role of GSTs in AML and to identify the exact GST responsible for the observed effects, a CRISPR/Cas9 loss-of-function screen of all 16 human *GST*s in an AML cell line combined with BCL-2 inhibitor treatment could be a promising approach.

Different studies have linked expression levels of ABC transporters - particularly *ABCB1*, *ABCC1* and *ABCG2* - with adverse prognosis in AML (M et al. 2005, Heuvel-Eibrink et al. 2007). Of these three ABC transporters, *ABCC1* was expressed at highest levels in our cohort of patient samples, followed by *ABCB1*. High expression of *ABCC1* was associated with poor disease-free survival in AML (Varatharajan et al. 2017). We found that *ABCG2* was not expressed in the AML samples examined by us. This is in line with previous findings of high expression of *ABCG2* in immature cell populations, like CD34+ cells, but only very low expression of *ABCG2* in AML blasts (Abbott et al. 2002). We also found that these findings - although obtained from a small patient cohort - match with the expression profile of ABC transporters of AML cells in the BeatAML dataset (Tyner et al. 2018). *ABCC1* was also the highest expressed ABC transporter among the examined members of the MRP subfamily. As *ABCC7*, *ABCC8* and *ABCC9* are not part of the MRP transporter subfamily and because they do not transport xenobiotic substrates and do not contribute to drug resistance their expression was not evaluated in this work. *ABCC11* and *ABCC12* expression was also not investigated, as these transporters were discovered as the last members of the ABCC subfamily and most likely do not play an important role in the hematopoietic system (Kruh et al. 2006). We did not detect expression of *ABCC2* and *ABCC6* in the examined AML patient samples. This was expected, as both *ABCC2* and *ABCC6* are expressed mainly in the liver and in other tissues that do not express *ABCC1* (Wang et al. 2021). Aside from *ABCC1*, only *ABCC3*, *ABCC4*, *ABCC5* and *ABCC10* were expressed in our cohort of patient samples. ABCC3 and ABCC10 are close homologs of ABCC1 and are both able to efflux xenobiotics, while ABCC4 and ABCC5 – besides acting as drug efflux pumps as well – also function as nucleoside transporters, which might be their role in the normal physiology of AML (Wang et al. 2021). The relatively high expression of *ABCC1* compared to all the other investigated ABC

transporters underlines the importance of this specific transporter in AML and could suggest that AML cells mainly rely on ABCC1 for detoxification.

We observed that the response of primary patient-derived AML samples to BCL-2 inhibition was mainly depended on the genetic features of the respective samples, with the samples harboring *FLT3* internal tandem duplications (P2, P4) being most resistant to Venetoclax. *FLT3*-ITD mutations are known mediators of primary resistance to Venetoclax (Chyla et al. 2018), as they enhance the expression of *BCL-X*$_L$ and *MCL-1* (DiNardo et al. 2020). In comparison to Venetoclax, treatment with AZD-4320 had a stronger effect in the *FLT3*-ITD positive samples (P2, P4), which is most likely due to the additional inhibition of BCL-X_L by this drug. Interestingly, the two samples which were already highly sensitive to Venetoclax (P1, P5) did not show increased sensitivity to AZD-4320, suggesting that they most likely depend on BCL-2 for cell survival. The two samples which were most sensitive to Venetoclax and AZD-4320 both had - amongst others - *DNMT3A* mutations (P1, P5). Patients with *DNMT3A* mutations were shown to have the highest rates of complete remission after Venetoclax treatment (DiNardo et al. 2020). Patient sample number 3 was not sensitive to Venetoclax, but to AZD-4320, but the underlying mutations or genotypes are not known for this specimen. Therefore, we cannot attribute these results to any molecular makeup. One thing to keep in mind when interpreting data of primary patient samples is that they are cultured in media supplemented with a variety of cytokines to sustain *in vitro* growth and delay differentiation, which could influence the response to drug treatments. This study was also limited by the small sample size (n=5) of primary patient-derived AML cells. With the exception of the samples which were already resistant to Venetoclax (P2, P3), combined treatment with Reversan potentiated the effect of Venetoclax and AZD-4320 (Fig. 16). First experiments using 5 µM Reversan resulted in increased sensitivity to Venetoclax and AZD-4320, but this concentration of Reversan had a slight impact on cell viability, suggesting that primary AML cells are more sensitive to ABCC1 inhibition compared to AML cell lines. By lowering Reversan concentrations to 2.5 µM for the remaining experiments, cell viability increased and was comparable to DMSO treated cells, while there was still a strong synergistic effect of combined ABCC1 and BCL-2 inhibition.

Even though our results clearly show that inhibition of ABCC1 potentiates the effect of BCL-2 inhibitors in cell lines as well as in patient samples, combinatorial treatment of ABCC1 and BCL-2 inhibitors is most likely not a therapeutic option for patients. Firstly, no ABCC1 inhibitor is currently approved for clinical use. Clinical trials using chemo-sensitizing agents targeting

ABCB1 failed due to side effects and lack of efficacy in the early 1980's (Cole 2014b). Some of the compounds that were tested inhibited ABCB1 and ABCC1, like Biricodar and Dofequidar (Cole 2014b). Despite the failure of ABC transporter inhibitors in clinical trials, the development of new, more specific inhibitors of ABCC1, the use of different delivery strategies or the use of antisense nucleotides could provide novel approaches in targeting ABCC1 more specifically. Besides the direct targeting of the transporter function, targeting of important co-factors like GSH could be another promising strategy, especially considering the synergistic effects of BSO and GST inhibitors with BCL-2 inhibition which we observed in this work.

As combined inhibition of ABCC1 and BCL-2 will unlikely be a treatment approach in the near future, our results could still propose *ABCC1* as a potential prognostic biomarker for the response to BCL-2 inhibition. Our RT-qPCR results showed that patients with a poor response to Venetoclax treatment had significantly higher *ABCC1* expression. Even though differences in the expression of other ABC transporters between patients with good and poor response were not significant, patients with a poor response to Venetoclax treatment showed a tendency for higher expression of most examined ABC transporters. Interestingly, co-expression of multiple ABC transporters is generally associated with a poor prognosis in AML (Liu et al. 2018) and could also explain why these patients showed a poor response to Venetoclax. As our analysis was limited by the small number of samples, the analysis of a bigger cohort of Venetoclax-treated patients from publicly available datasets like the BeatAML (Tyner et al. 2018) dataset could further evaluate whether *ABCC1* expression could be a potential prognostic biomarker for Venetoclax response in AML.

The most plausible explanation for all our findings is that Venetoclax and AZD-4320 are substrates of ABCC1 and are effluxed via a GSH-dependent mechanism. Hence, genetic or pharmacological inhibition of ABCC1 leads to an accumulation of Venetoclax and AZD-4320 in AML cells, resulting in increased drug toxicity. To confirm that this is the mechanism behind the observed effect, mass spectrometry experiments could be used to determine the differences between the intracellular concentrations of Venetoclax or AZD-4320 in cells expressing functioning ABCC1 versus cells with impaired ABCC1 function. Additionally, *in silico* docking studies could also predict interactions between the two compounds and ABCC1. Unfortunately, the crystal structure of human ABCC1 is still not completely resolved, and only the molecular structure of bovine Abcc1, which shares 91% homology with ABCC1 - has been determined so far (Johnson und Chen 2017). Moreover, virtual profiling of interactions between

substrates and transporters is difficult, as transporters often contain multiple drug binding pockets and undergo substantial conformational changes after binding substrates or ATP.

It should not be dismissed that inhibition of ABCC1 already changes physiological processes including redox homeostasis and GSH metabolism. These changes might not be enough to induce cell death under normal conditions, but still could render AML cells more susceptible to BCL-2 inhibition. As ABCC1 has also been detected in the mitochondria (Roundhill and Burchill 2012), inhibition of ABCC1 could also lead to changes in the mitochondria, potentially leading to increased BAX/BAK oligomerization or increased mitochondrial outer membrane permeabilization. Even though the above-mentioned mechanisms could partially contribute to the observed effects of increased BH3-mimetic sensitivity in AML cells with impaired ABCC1 function, the most likely scenario is still that the impairment of ABCC1 leads to higher intracellular drug concentrations, resulting in increased toxicity of BCL-2 inhibitors.

Taken together, the results of this work identify ABCC1 as an important interactor in drug response of AML cells and inhibition of ABCC1 and perturbation of GSH homeostasis increases the sensitivity of AML cells to the BH3-mimetics Venetoclax and AZD-4320.

5. Abstract

Acute myeloid leukemia (AML) is an aggressive hematological malignancy caused by the clonal expansion of undifferentiated myeloid precursors. Standard treatment of AML is induction chemotherapy consisting of Cytarabine and Daunorubicin. AML has a poor prognosis, especially for elderly patients. Several targeted therapies – including the BCL-2 inhibitor Venetoclax - have been recently approved for AML-treatment, thereby expanding the treatment options for older patients who are unfit for intensive chemotherapy.

The aim of my work was to investigate the influence of the Multidrug Resistance Associated Protein 1 (MRP1, ABCC1) on Venetoclax response in human AML cell lines. ABCC1 is a member of the ATP Binding Cassette (ABC) transmembrane transporter family that has been associated with drug resistance in cancer, as it can efflux chemotherapeutic drugs like Etoposide or Daunorubicin. RT-qPCR analysis of patient samples revealed that *ABCC1* was among the highest expressed ABC transporters in AML. CRISPR/Cas9 mediated knockout of *ABCC1* in AML cell lines sensitized them to Venetoclax and to the structurally related BCL-2/ BCL-X_L inhibitor AZD-4320. Similarly, shRNA-mediated knockdown of *ABCC1* in various cell lines sensitized AML cells to BCL-2-inhibiton. Combinatorial treatment of BH3-mimetics and the ABCC1 inhibitor Reversan resulted in synergistic effects in AML cell lines and primary patient-derived AML cells. To further corroborate these findings, the role of glutathione (GSH) - which is known to play an important role in ABCC1 mediated drug efflux – was investigated. Inhibition of glutathione synthesis using the Gamma-glutamylcysteine synthetase inhibitor Buthionine Sulfoximine as well as inhibiting GSH-drug-conjugation using glutathione-S-transferase inhibitors (Ethacrynic acid, Ezatiostat), increased the sensitivity of AML cells to Venetoclax.

Taken together, these results demonstrate that ABCC1 perturbation sensitizes AML cells to BCL-2-inhibiton and that GSH plays an important role in modulating Venetoclax sensitivity. This work deepens our understanding of the interaction between ABCC1, BCL-2-inhibitors and glutathione.

6. Zusammenfassung (Deutsch)

Die akute myeloische Leukämie (AML) ist eine aggressive Erkrankung des blutbildenden Systems, hervorgerufen durch die klonale Vermehrung unreifer myeloischer Vorläuferzellen. Die meisten Patienten erhalten eine Induktions-Chemotherapie bestehend aus Cytarabine und Daunorubicin. AML hat immer noch eine schlechte Prognose, besonders für ältere Patienten. In den letzten Jahren wurden mehrere zielgerichtete Therapien – unter anderem der BCL-2 Inhibitor Venetoclax – für die Behandlung von AML zugelassen und erweitern somit die Behandlungsoptionen für ältere Patienten.

Das Ziel meiner Arbeit war es, den Einfluss von ABCC1 auf die Wirkung von Venetoclax in humanen AML Zelllinien zu erforschen. ABCC1 (Multidrug Resistance Associated Protein 1, MRP1) gehört zur Familie der ATP binding cassette (ABC) Transporter und wurde mit Arzneimittelresistenz in Krebspatienten assoziiert, da ABCC1 Chemotherapeutika wie Etoposide und Daunorubicin aus den Zellen transportieren kann. RT-qPCR Analyse von Patientenproben zeigte, dass ABCC1 zu den am höchsten exprimierten ABC Transportern in AML gehört. Knockout von *ABCC1* mittels dem CRISPR/Cas9 System sensibilisierte verschiedene AML Zelllinien auf Venetoclax und den strukturell verwandten BCL-2/ BCL-X_L Inhibitor AZD-4320. Vergleichbare Ergebnisse konnten mittels shRNA-basiertem Knock-down von *ABCC1* in verschiedenen AML Zelllinien mit BCL-2 Inhibitoren erzielt werden. Gleichzeitige Behandlung mit BCL-2 Inhibitoren und dem ABCC1 Inhibitor Reversan zeigten synergistische Effekte in AML Zelllinien und primären Zellen von AML Patienten. Um unsere Ergebnisse näher zu erforschen, wurde die Rolle von Glutathion (GSH) – von dem bekannt ist, dass es eine wichtige Rolle im Transport von Xenobiotika durch ABCC1 spielt – erforscht. Inhibition der GSH Synthese durch den Gamma-Glutamylcysteine Synthetase Inhibitor Buthionine Sulfoximine und Inhibition der Konjugation von GSH an Arzneimittel durch Glutathion-S-Transferase Inhibitoren (Ethacrynsäure, Ezatiostat), erhöhte die Empfindlichkeit von AML Zellen auf Venetoclax.

Zusammenfassend zeigen diese Ergebnisse, dass die Beeinträchtigung von ABCC1 AML Zellen empfindlicher gegen BCL-2 Inhibitoren reagieren lässt. Des Weiteren spielt GSH eine wichtige Rolle in der Modulation der Potenz dieser Inhibitoren. Diese Ergebnisse vertiefen unser Verständnis über die Interaktion zwischen ABCC1, BCL-2 Inhibitoren und GSH.

7. Abbreviations

AAVS1	adeno-associated virus integration site 1
ACTB	ß-ACTIN
ABC	ATP binding cassette
ALL	acute lymphocytic leukemia
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
ATO	arsenic trioxide
ATRA	all trans retinoic acid
BAK	BCL-2-associated X protein
BAX	BCL-2 antagonist killer 1
BCL-2	B-cell lymphoma 2
BCL-X_L	B-cell lymphoma-extra large
BSO	Buthionine Sulfoximine
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
CR	complete remission
CRi	complete remission with incomplete hematologic recovery
DNMT3A	DNA methyltransferase 3 alpha
FLT3	FMS-like tyrosine kinase 3
GCL	Glutamate-cystein ligase
GSH	glutathione
GSS	Glutathione synthetase
GSSG	glutathione disulfide
GST	Glutathione-S-transeferase
HiDAC	high dose Cytarabine
HSCT	hematopoietic stem cell transplant
IRFP	near-infrared fluorescent protein
IDH1	Isocitrate dehydrogenase 1
IDH2	Isocitrate dehydrogenase 2
INDEL	Insertion-deletion
ITD	internal tandem replication
JNK	c-Jun N-terminal kinase
LDAC	low dose Cytarabine
LTC_4	leukotriene C_4
MCL-1	Myeloid cell leukemia 1
MDR	multiple drug resistance
MRP1	multiple resistance associated protein 1
MSD	membrane spanning domain
n.d.	not determined
n.s.	not significant
NBD	nucleotide binding domains

RNAi	RNA interference
RPL17	60S ribosomal protein L17
sgRNA	single guide RNA
shRNA	short hairpin RNA
SLL	small lymphocytic lymphoma
SMO	Smoothened
TIDE	tracking of INDELS by decomposition
TKD	tyrosine kinase domain

8. References

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9. List of figures

10. List of tables

Appendix

Table 6: List of used primers in this work.

Use	Target	Forward primer	Reverse primer
Genotyping	sgABCC1.1 target region	CCCTGAAGGGTGACATTCCC	CTGCTGGCATTTCCTTGCTC
Genotyping	sgABCC1.2 target region	GCCGGCTTTCTGCCATTACA	CTGCAAGACAGCAGTTCATCAAT
RT-qPCR	BCL-2	CTGCACCTGACGCCCTTCACC	CACATGACCCCACCGAACTCAAAGA
RT-qPCR	BCL-X_L	GGAGAACGGCGGCTGGGATA	GGCCACAGTCATGCCCGTCA
RT-qPCR	MCL-1	AGAAAGCTGCATCCAACCAT	CCAGCTCCTACTCCAGCAAC
RT-qPCR	ABCB1	GGAAAGTGCTGCTTGATGGC	AGGCATGTATGTTGGCCTCC
RT-qPCR	ABCG2	CATGGTGTATAGACGCCCTGAC	GTTCCCAAATTGATGTTGTGACAGA
RT-qPCR	ABCC1	TTACTCATTCAGCTCGTCTTGTC	CAGGGATTAGGGTCGTGGAT
RT-qPCR	ABCC2	TCCTTTGCAAGTGACCGTGA	CCTTCCTGGCCAAGTTGGAT
RT-qPCR	ABCC3	CCTTTGCCAACTTTCTCTGC	AGGGCACTCAGCTGTCTCAT
RT-qPCR	ABCC4	CATGACTTGGACACGGTAACTGTTG	TCAGGAATGTCGGTTAGAGGTTTG
RT-qPCR	ABCC5	GGAGCTCTCAATGGAAGACG	CACACGATGGACAGGATGAG
RT-qPCR	ABCC6	CACAGTTTGTGCTGTCCTGC	CCAAGCGACCAGAGGTCTTT
RT-qPCR	ABCC10	AAACCAGAGGTGCCAGTTTG	TGGCCTCTGTCTGTGTGAAG

Table 7: List of the sgRNA sequences used in this work

Target	Sequence
AAVS1.1	GCTCCGGAAAGAGCATCCT
AAVS1.2	GCTGTGCCCCGATGCACAC
RPL17.1	GTACCATTCCGACGTTACAA
RPL17.2	GACATCTTTCAGATACTTCG
ABCC1.1	GTATAACACCTTAAACAGAG
ABCC1.2	GGATGGTTTCCGAGAACAG
ABCC1.3	GGGCTGACCAGAAACACTG

Table 8: List of the shRNA sequences used in this work

Target	Sequence
Renilla	TGCTGTTGGCAGTGAGCGCAGGAATTATAATGCTTATCTATAGTGAAGCCACAGATGTATAGATAAGCATTAT AATTCCTATGCCTACTGCCTCGGA
RPL17.1	TGCTGTTGGCAGTGAGCGAACGGGAGTAAATTCAGCATTATAGTGAAGCCACAGATGTATAATGCTGAATTT ACTCCCGTGTGCCTACTGCCTCGGA
RPL17.2	TGCTGTTGGCAGTGAGCGAAGAAGAAACTGAAGAAACAAATAGTGAAGCCACAGATGTATTTGTTTCTTCAG TTTCTTCTGTGCCTACTGCCTCGGA
ABCC1.1	TGCTGTTGGCAGTGAGCGCGGAGAACTTGATATTTAGTTATAGTGAAGCCACAGATGTATAACTAAATATCAA GTTCTCCATGCCTACTGCCTCGGA
ABCC1.2	TGCTGTTGGCAGTGAGCGCGGTTATGAAGCTTTCAAAGTATAGTGAAGCCACAGATGTATACTTTGAAAGCT TCATAACCATGCCTACTGCCTCGGA
ABCC1.3	TGCTGTTGGCAGTGAGCGCACCATTCTTTCACATTAGATATAGTGAAGCCACAGATGTATATCTAATGTGAAA GAATGGTATGCCTACTGCCTCGGA