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PROBING INHIBITORY POTENTIAL OF ARYL-METHOXY DERIVATIVES ON ABCG2 WITH A FLUORESCENT SUBSTRATE ACCUMULATION ASSAY

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Dedicated to my family and friends

I would like to express my sincere appreciation of, and heartful thanks to all members of the MMCT Laboratory who supported me over 5 months, especially my co-supervisors Anna Cseke and Katrin Wlcek for their guidance in all matters. I also want to thank my supervisor Prof. Ecker and Prof. Ogris for facilitating me to join their laboratories and for the friendly support.

Table of Contents

Abstract

The ATP binding cassette (ABC) transporter superfamily is one of the largest protein families as its members are expressed ubiquitously in prokaryotic and eukaryotic organisms. They are transmembrane proteins using the energy derived from ATP hydrolysis to pump substrates across the membrane against the concentration gradient.

Breast Cancer Resistance Protein (BCRP / ABCG2), a member of the ATP binding cassette family, is an efflux transporter which plays a crucial role in the development of multidrug resistance (MDR) against anticancer drugs. It is overexpressed in the cell membrane of several tumor cell types and mediates the drug efflux out of the cells. This leads to a reduction in the intracellular cytostatic concentration and subsequently, to failure of cancer chemotherapy.

Physiologically, BCRP is highly expressed in the small intestine, kidney, liver, lungs, brain endothelium, mammary glands and placenta, and it protects these tissues from a variety of endogenous and exogenous toxins by extruding them from the cells. Due to its location in the physiological barriers and its substrate polyspecificity, it affects absorption, distribution, and elimination of drugs, which have an influence on the oral bioavailability and tissue distribution. Furthermore, BCRP is also involved in drug-drug interactions.

In this work, a set of aryl-methoxy derivatives was investigated for their BCRP inhibitory potential, based on predictions from docking studies on a homology model of BCRP. Four compounds were examined for their inhibition of BCRP mediated mitoxantrone efflux in BCRP-expressing PLB985 cells, using flow cytometric analysis. Our aim was to gain a deeper knowledge about the molecular mechanisms of BCRP inhibition. Our results have shown that all four compounds have an inhibitory effect on BCRP, which was likely due to their H-bond interaction with Glutamate 73 at the binding site of BCRP.

Zusammenfassung

Die Superfamilie der ATP-Bindungskassette (ABC) ist eine der größten Proteinfamilien, da ihre Mitglieder in prokaryotischen und eukaryotischen Organismen ubiquitär exprimiert werden. Es handelt sich um Transmembranproteine, die aus der ATP-Hydrolyse gewonnene Energie verwenden, um Substrate über die Membran gegen den Konzentrationsgradienten zu pumpen.

Brustkrebsresistenz-Protein (BCRP / ABCG2), ein Mitglied der ATP-Bindungskassettenfamilie, ist ein Efflux-Transporter, der bei der Entwicklung von Multidrug-Resistenzen (MDR) gegen Antikrebs-Medikamente eine entscheidende Rolle spielt. Es wird in der Zellmembran von mehreren Tumorzelltypen überexprimiert und vermittelt den Wirkstoffabfluss aus den Zellen. Dies führt zu einer Verringerung der intrazellulären zytostatischen Konzentration und anschließend zum Versagen der Krebs-Chemotherapie.

Physiologisch wird BCRP im Dünndarm, in der Niere, in der Leber, in der Lunge, im Gehirnendothel, in den Brustdrüsen und in der Plazenta stark exprimiert und schützt diese Gewebe vor einer Vielzahl von endogenen und exogenen Toxinen, indem sie aus den Zellen extrudiert. Aufgrund seiner Lage in den physiologischen Barrieren und seiner Substratpolyspezifität beeinflusst sie die Absorption, Verteilung und Beseitigung von Arzneimitteln, die einen Einfluss auf die orale Bioverfügbarkeit und Gewebeverteilung haben. Darüber hinaus ist BCRP auch an Arzneimittel-Wechselwirkungen beteiligt.

In dieser Arbeit wurde ein Set von Aryl-Methoxy-Derivaten für ihr BCRP-inhibitorisches Potential untersucht, basierend auf Vorhersagen von Docking-Studien an einem Homologie-Modell von BCRP. Vier Verbindungen wurden auf ihre Hemmung des BCRP-vermittelten Mitoxantron-Efflux in BCRP-exprimierenden PLB985-Zellen unter Verwendung einer Durchflusszytometrie untersucht. Unser Ziel war es, ein tieferes Wissen über die molekularen Mechanismen der BCRP-Hemmung zu gewinnen. Die Ergebnisse haben gezeigt, dass alle vier Verbindungen eine hemmende Wirkung auf BCRP haben, was wahrscheinlich aufgrund ihrer H-Bindungs-Wechselwirkung mit Glutamat 73 an der Bindungsstelle von BCRP war.

1. INTRODUCTION

1.1 ATP-binding cassette transporters

The superfamily of the ATP-binding cassette (ABC) transporters is a large family of transmembrane proteins whose members are present in all eukaryotic and prokaryotic organisms [1]. In mammals and humans, most of these transporters are expressed in cellular membranes. In human, there are 49 known ABC transport proteins which are divided into seven different subfamilies from ABCA through to ABCG based on their sequence similarities [2]. They use the energy of ATP hydrolysis to transport diverse substrates including peptides, sugars, amino acids, phospholipids, sterols and metabolites across extra- and intracellular membranes [3, 4].

Most of the ABC transporters consists of four domains, two transmembrane domains (TMD) and two nucleotide binding domains (NBD) [5]. The transmembrane domains contain hydrophobic segments for the anchorage in the phospholipid membrane and consist of typically 6 membrane spanning alpha helices which are responsible for the substrate recognition and translocation across the cell membrane [6]. The NBDs are located in the cytoplasm and characterized by typical sequence motifs called Walker A, Walker B, and an ABC signature motif which have an essential role in the hydrolysis of ATP to ADP+Pi and energy coupling. The binding of two ATP molecules is necessary for NBD dimerization to form the ATP-sandwich NBD dimer. In mammals, the four core domains can be present in the same polypeptide chain (‘full transporters’) or two separate polypeptide chains (‘half transporters’). In the case of ‘half transporters’, for the formation of a functional ABC transporter the protein needs to homo- or heterodimerize [7, 8].

The general mechanism for the transport cycle of ABC transporters has not been completely clarified, but it is known that the drug extrusion comprises ATP binding and hydrolysis linked to conformational changes in the nucleotide binding domain [9]. The transport cycle initiate the drug binding to a high-affinity pocket, which is modeled by the TMDs. After that a conformational change is induced in the NBDs, enabling ATP binding and the formation of the closed NBD dimer. The releasing energy from the formation causes conformational changes in the TMDs which opens the drug binding site toward the outside and induce substrate translocation. Subsequently, ATP hydrolysis facilitate

dissolution of the closed NBD dimer and return the transporter to the open NBD-dimer conformation after releasing of Pi and ADP. (Figure1.)

The ATP hydrolysis only proceeds if both catalytic sites are functional [10].

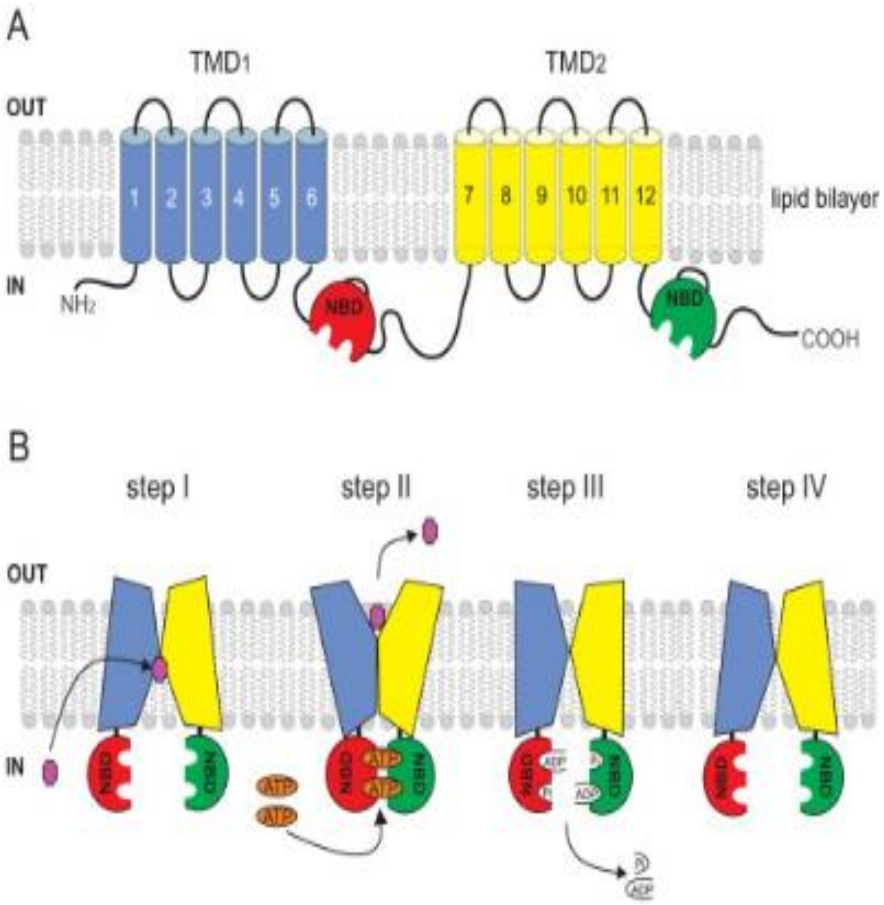


Figure1.

A) Typical structure of an ABC full transporter containing two TMDs, each containing 6 transmembrane (TM) segments, and two NBDs. B) ATP-switch model for the transport cycle of an ABC transporter [8].

1.2 Breast Cancer Resistance Protein (BCRP)

The Breast cancer resistance protein (BCRP) is the second member of the G subfamily of the ABC transporter superfamily (ABCG2). Driven by the energy of ATP hydrolysis, BCRP pumps out varied substrates from the cell through the membrane [4].

BCRP was discovered in the drug resistant breast cancer cell line MCF-7/AdrVp and therefore it got its name "Breast Cancer Resistance Protein". It mediates resistance to different chemotherapeutic agents as methotrexate, doxorubicin and mitoxantrone by pumping these compounds out of the cell [11]. In addition to chemotherapeutics, there are several compounds from different drug classes as well as fluorescent dyes which have also been shown to be transported by BCRP [12]. (Table 1.)

Table 1. BCRP substrates

Class of drugs	**Compounds**
Anthracenes	Mitoxantrone Bisantrene
Campothecins	Irinotecan Topotecan
Anthracyclins	Doxorubicin Daunorubicin
Antimetabolite	Metotrexate
Fluorescent dyes	Hoechst 33342 BODIPY-prazosin Rhodamin 123

BCRP is a half ABC transporter, and contains only one transmembrane domain at the C-terminal end and one nucleotide binding domain at the N-terminal end. BCRP forms homodimers to be functionally active as an efflux protein [13]. (Figure 2.)

The BCRP transporter is expressed in the apical plasma membrane in tissues with physiological barrier function, including the proximal tubule cells in the kidney, the capillary endothelial cells of the blood-brain barrier, the enterocytes in the gastrointestinal tract, and the hepatocytes in the liver [12, 14]. BCRP plays a major role in changing the pharmacokinetics of transported drugs, for instance absorption, distribution, and excretion [15]. This has an impact on the oral bioavailability and tissue distribution. Also, BCRP is involved in drug-drug interactions: while the transport activity

of BCRP can reduce the bioavailability of certain drugs, BCRP inhibition by another substance increases it [16].

Besides its role in the physiological barriers, the high expression level of BCRP in solid tumors like breast cancer, gastric carcinoma and intestinal cancers can be a major factor in the development of multidrug resistance (MDR) [17].

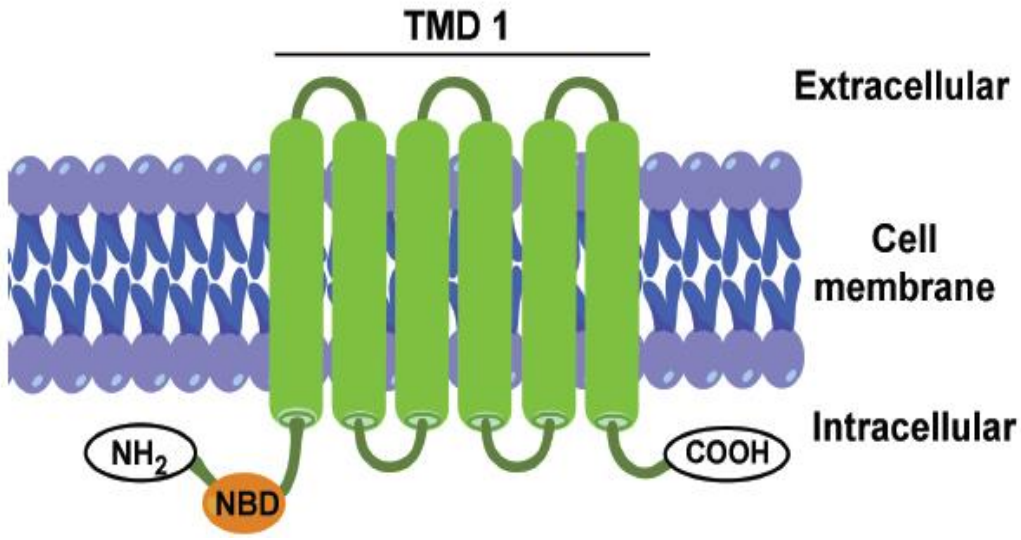


Figure2.

Diagram of ABCG2 showing the half-transporter structure with six transmembrane segments and one NBD at the N-terminal end[18].

1.3 Chemotherapy and multidrug resistance (MDR)

Over the last decades, chemotherapy has been an important form of treatment for different types of cancer.

One of the major reasons of the failure of chemotherapy is the resistance against multiple anticancer agents, which is called multidrug resistance (MDR). Multidrug resistance, a phenomenon seen in tumor cells, is a mechanism by which cells or organisms develop resistance toward a wide spectrum of structurally and functionally different drugs [19, 20].

Generally, multidrug resistance is classified as either intrinsic or acquired. An intrinsic resistance is genetically determined, in this case it occurs at the beginning of the therapy. Acquired resistance develops during the therapeutic treatment. The increased expression level of the ATP binding cassette (ABC) transporters, ABCB1, ABCC1 and ABCG2, also called as MDR transporters, often leads to the failure of the chemotherapy [21]. These ABC proteins extrude the anticancer drugs out of the cells and thereby prohibit their interaction with their intracellular targets. Overexpression of MDR transporters considered to play a role in the development of multi drug resistance of some hematologic disorders such as acute myeloid leukemia (AML) and certain solid tumors. This has been demonstrated in vitro based on various tumor cell lines [22].

The high expression of BCRP is a characteristic feature of cancer stem cell-like side population which have the ability to self-renew and differentiate. Side populations have been recognized in several human tissues including lung, muscle, mammary glands, liver and in many tumors and cancer cell lines. These cancer side population cells show various properties referable to stem cell-like cancer cells and have been involved in tumor growth, and metastasis [23].

1.4 BCRP inhibitors

BCRP is involved in the development of multidrug resistance during the chemotherapy and is thus regarded as an obstacle in cancer treatment. Therefore, a major effort has been made to establish specific BCRP inhibitors. It was expected that on one hand, the inhibition of BCRP in cancer cells increases the intracellular cytostatic concentration and induces the death of malignant cells [24]. On the other hand, the inhibition of BCRP enhances the bioavailability and plasma concentration of drugs which are also BCRP substrates. In an attempt to eradicate the multidrug resistance by inhibiting the ABC efflux pumps, various inhibitors of BCRP have been developed.

Certainly, this attempt failed, although many nominees entered auspiciously into the preclinical phase [18, 25]. Many explanations have been submitted why such inhibitors failed to provide a positive effect in the clinical phase. One implied that many of the candidates cannot target tumor cells and thereby get every single cancer cell. The reduced permeability and enzymatic deactivation of anticancer agents also limit the effectiveness of chemotherapy [26]. Also, the unwanted side effects and pharmacokinetic complications make it difficult to obtain successful inhibitors.

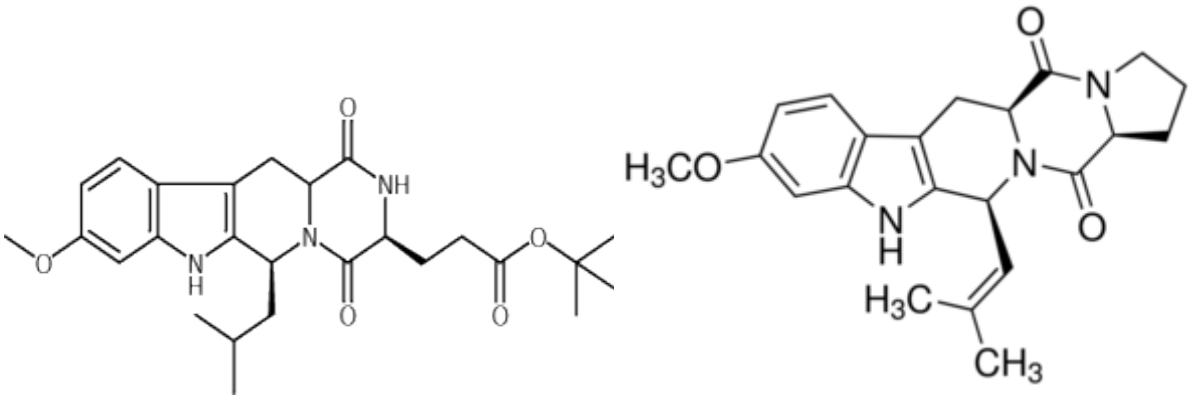
However, it is still interesting to investigate the molecular mechanism of BCRP inhibition to get a better understanding about BCRP mediated drug-drug interactions that might induce alterations in the pharmacokinetics of drugs [27]. Also, the knowledge about the relationship between the chemical structure of compounds and their inhibitory effect on BCRP may contribute to the development of new potent inhibitors.

One of the first high potent and specific BCRP inhibitors was Fumitremorgin C (FTC), a tremorgenic mycotoxin isolated from the fungus *Aspergillus fumigatus* (Figure3.). However, because of its neurotoxicity it has a limited application *in vivo*. The most potent analogue of FTC is Ko143 (Figure 3.). It has a higher BCRP inhibitory effect and lower *in vivo* cytotoxicity than FTC. Ko143 inhibits BCRP selectively in nanomolar concentrations (5-10 nmol/l) and reverses BCRP mediated drug efflux. The methoxy group on the aromatic indole ring plays an important role in the increased BCRP inhibitory activity [28].

A couple of naturally occurring flavonoids such as 6 prenyl-chrysin and bioachanin A have been investigated for their inhibitory effect on BCRP. It was reported that they

significantly increased mitoxantrone accumulation in BCRP-overexpressing cells. The high inhibitory effect and the low cytotoxicity of flavonoids make them very interesting as BCRP inhibitors in the cancer treatment [29].

Tyrosine kinase inhibitors are used against malignant cells and metastasis, and have also been proposed to interfere and inhibit BCRP by blocking the phosphorylation mediated by tyrosine kinase and the binding of ATP. The inhibition of tyrosine kinase interrupt several cellular functions and stops the signal transduction pathway. It induces a decreased cell proliferation and apoptosis. Some compounds of tyrosine kinase inhibitors (Imatinib, Bafetinib) enhance the intracellular accumulation of mitoxantrone in BCRP overexpressing cells [30, 31].



Ko143 **Fumitremorgin C (FTC)**

Figure3.
Structure of Ko143 and Fumitremorgin C.

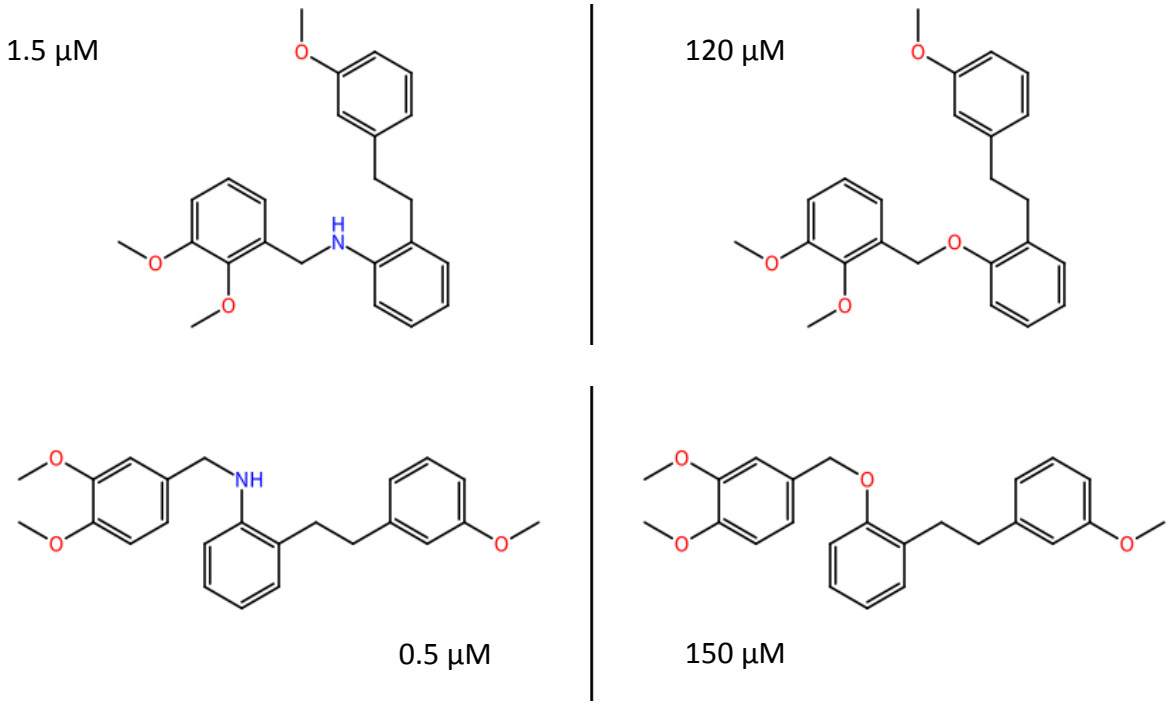
2. Objectives of the work

In the last years, various BCRP inhibitors have been developed and progressed in the purpose to eradicate the multidrug resistance due the overexpressing of BCRP in diversity of cancer cells.

The aim of the thesis was to test the inhibitory effect of aryl-methoxy derivatives for BCRP *in vitro* and thereby, to gain insight into the mechanisms of BCRP inhibition. These compounds were synthesized followed by a homology modeling and docking. In this work four test compounds were investigated for their BCRP inhibition as well as the influence of their physicochemical properties to the obtained results. The homology modeling and docking was made by Floriane Montanari (supervisor: Prof. Gerhard Ecker) and the synthesis of the aryl-methoxy derivatives by Giovanne Parisi and George Stanchev (supervisor: Prof. Vittorio Pace).

The basis of this work was the publications of Colabufo et al [32-34]. For our BCRP docking studies four compounds from one of the publications (Colabufo et al 2008a) were used: two of them showed good BCRP inhibition (mol61 and mol62, IC50 values of 1.5μM and 0.5μM, respectively) and two were less active (mol69 and mol27, IC50 values of 120μM and 150μM, respectively) (Figure 4A.). We found a binding mode where the two compounds with amine linker are forming a H-bond with Glutamate73 in BCRP. The other 2 compounds with the ether linker are not making this interaction (Figure 4B.).

A)



B)

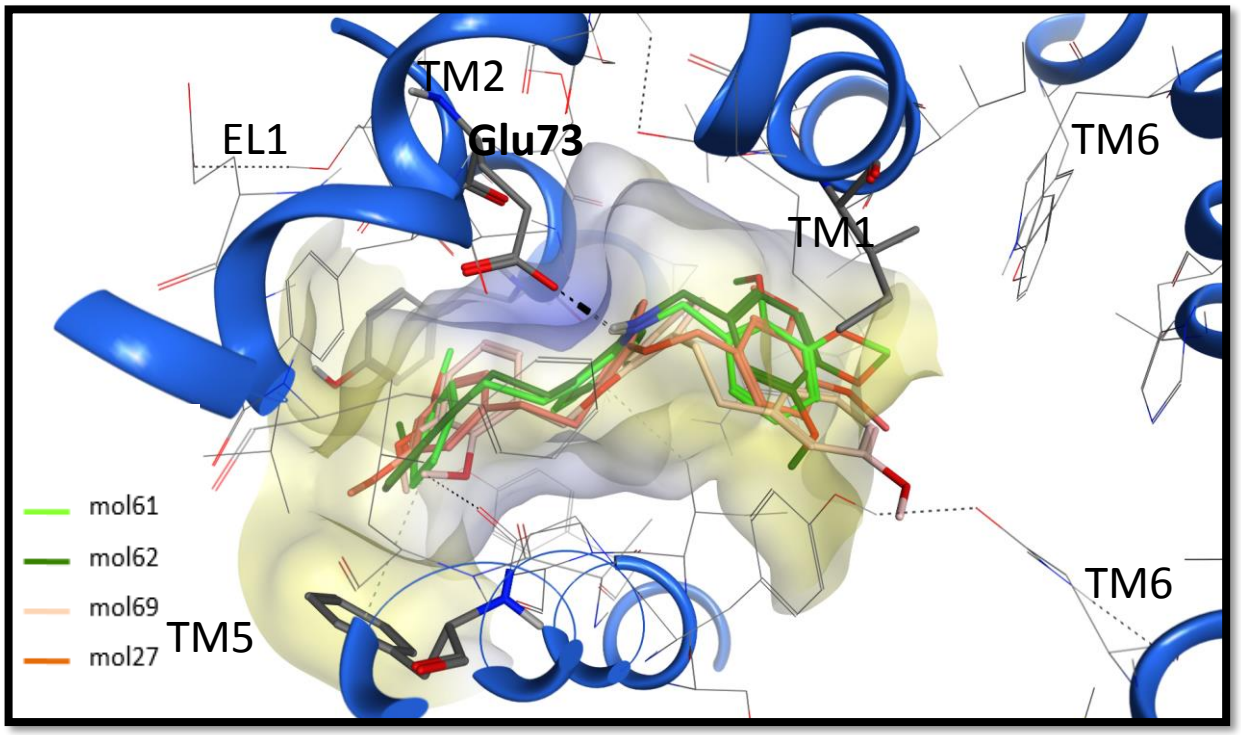


Figure4.

A) Structure of (hetero)arylmethyloxy-and arylmethylamine-phenyl derivatives. The replacing of an amine linker by an ether linker induces a loss of inhibitory activity against BCRP[34]. **B)** Binding hypothesis model made by Floriane Montanari. A binding mode was found in the protein where two compounds with amine group are making an H-bond with Glutamate73 while the other two compounds don't make this interactions.

For this work, four test compound were synthesized (GP199-1, GP196-2, GS3, GS4) to prove the hypothesis that a H-bond to Glutamate 73 is necessary for BCRP inhibition in aryl-methoxy derivatives (Figure5.).

The prediction for our test compounds was that the two with hydroxyl-groups should be able to form a H-bond with the Glutamate 73, while the two with ketone-groups should not.

The BCRP protein have many aromatic and hydrogen bond donor side chains. It was previously reported that the methoxy and hydroxyl groups have an influence on the inhibitory potency depending on localization of the group in the structure [27].

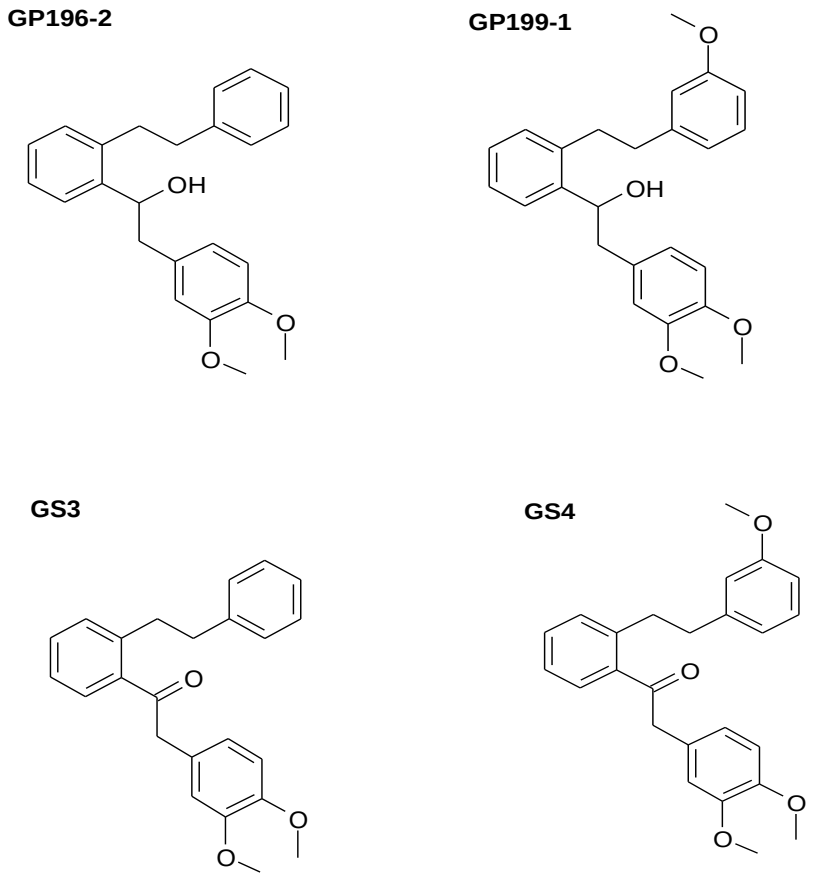


Figure 5.
Structure of the arylmethoxy-derivatives, newly synthesized to test BCRP inhibition efficiency.

3. Materials and methods

3.1 Materials

- 25 cm^2 , 75 cm^2 , 175 cm^2 cell culture flasks
- Pipettes 5ml, 10ml, 25ml
- Pasteur pipettes
- Pipette tips (nerbe plus 10μl, 200μl, 1250μl)
- 5ml Non-sterile FACS tubes
- 15ml, 50ml centrifugation tubes
- 1.5ml Microcentrifuge tubes (Eppendorf tubes)
- Ice box (styrofoam)
- Cryovials containing frozen cells
- RPMI 1640 medium (Gibco-Ref: 52400-025) with L-glutamine,
- FCS (Fetal Calf Serum) (SIGMA F7524, Lot No: 014M3395)
- PBS (Phosphate buffered saline pH 7.4)
- HPMI (HEPES-buffered medium RPMI-1640)
- DMSO (Dimethyl-sulfoxide) (D5879 Sigma-Aldrich)
- Fluorescent stain DAPI (4',6-diamidino-2-phenylindole) (D9542 Sigma-Aldrich)
- Mitoxantrone (M6545 Sigma-Aldrich)
- Ko143 (K2144 Sigma-Aldrich)

3.2 Devices

- Incubator (Thermo Scientific Heracell 150i CO2 Incubator)
- Laminar flow cabinet (Thermo Scientific Herasafe KS)
- Water bath (VWB 18)
- Centrifuge (HERAEUS Megafuge 16R)
- Vortex mixer (VELP Scientifica)

- Microscope (BRESSER GmbH-Motic-AE 31)
- Flow cytometer (MACSQuant; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany)
- Thermomixer (Eppendorf)

3.3 Buffers

☐ HPMI buffer

In this work all cell test including mitoxantrone accumulation measurement and DMSO testing were carried out in HPMI buffer. HPMI is also isotonic, the pH is physiologic and it contains glucose that the cells stay alive longer.

Table 2. Composition of HPMI buffer (pH 7.4)[35]

Substance	**Molarity [mM]**
Sodium chloride	120
Potassium chloride	5
Magnesium chloride	0.4
Calcium chloride	0.04
D-glucose monohydrate	10
HEPES	10
Sodium bicarbonate	10
Sodium hydrogen phosphate	5

☐ Phosphate buffered saline (PBS)

PBS is a buffer solution which is widely used in biological assays. Because of its isotonic and nontoxic properties, it is very convenient for washing cells.

Table 3. Composition of PBS (pH 7.4)

Substance	Molarity [mM]
Sodium chloride	137
Potassium chloride	2.7
Monopotassium phosphate	2
Disodium phosphate	8

3.4 Cultivation of cell lines

The human acute myeloid leukemia PLB985 parental and the stable BCRP cell lines were received from B. Sarkadi (Institute of Enzymology, Research Centre for Natural Sciences, Budapest, Hungary) and K. Nemet (Creative Cell Ltd., Budapest, Hungary). Acute myeloid leukemia is a type of cancer identified by the clonal expansion of leukemic myeloid cells. Both cell lines were cultured in RPMI 1640 completed with 10 % FBS. Cell culture was passaged two or three times a week to sustain the cell density between 0.2*10^6 and 1.5*10^6/ml. All cell lines were cultivated in an incubator at 37°C and 5 % CO_2 with 95 % relative humidity.

All used cell lines in this study were handled aseptically in a hood with laminar air flow in standard culture flasks to avoid a contamination with microorganisms. Likewise, all objects that are placed in the laminar air flow, were previously cleaned with 70% ethanol. Tissue culture flasks of different size T25(25cm^2), T75(75cm^2), T175(175cm^2) were used depending on the needed number of cells for the assay. Before starting the cultivation, the culture medium was heated in a water bath at 37°C to reach the optimal temperature.

3.4.1 Cryopreservation of cells

Cryopreservation, also called cryoconservation is a procedure where cells, tissues or other biological structures are preserved by freezing at -150°C. At this low temperature enzymatic activities that might affect the cells harmfully are blocked. With the aid of this method, it is possible to maintain the vitality of the cells almost unlimited and can be obtained over a long period of time. Cells were harvested and after centrifugation the cell pellet was re-suspended in cryomedium (70% RPMI, 20% FCS, 10% DMSO) and was transferred into cryovials. DMSO were added to prevent a crystal formation during freezing the cells. Finally, the cells were frozen and stored at -150°C.

3.5 Flow cytometry

The flow cytometry, also called Fluorescence Activated Cell Sorter (FACS) is a technique used to measure the fluorescence intensity as well as the size, and granularity of as they pass in a fluid stream throughout a ray of laser light at varied wavelengths. The measurement process is based on the principle of hydrodynamic focusing. It is a technique to generate a tight fluid stream containing cells moving in a single line through a flow channel and thus facilitating to determine the size of individual particles.

Flow cytometry is used in a wide range of applications such as immunophenotyping , cell counting, cell sorting or clinical trials. A flow cytometer is basically composed of three functional objects: a fluid system, an optical system and a detector which measures the light intensity. At the process the examined cell suspension will be injected through a cannula into a flow sheath fluid. The individualized cells are detected as they pass through the laser beam [36]. Cells are characterized by the following parameters:

- Forward-scattered light (FSC) is proportional to the cell surface and measures the size and the shape of the cells.

- Sideward-scattered light (SSC) is generated by refraction and reflection of light, it is collected at 90 degrees to the laser path and measures the granularity of the cell.

An important point is that they are also characterized by the intensity of the emitted fluorescence at different wavelengths. This is the advantage that several wavelengths can be detected at the same time with different detectors.

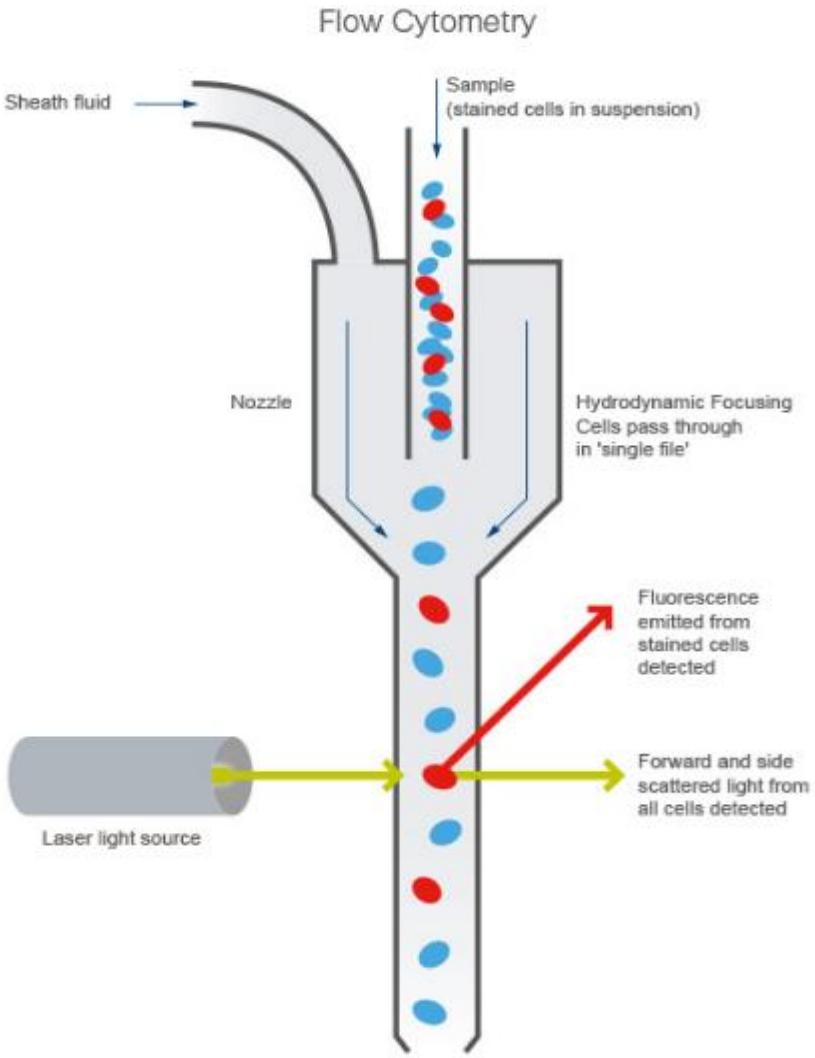


Figure5.

Scheme of the flow cytometer. Sheath fluid focuses the cell suspension, causing cells to pass through a laser beam. Forward and side scattered light is detected, as well as fluorescence emitted from stained cells [37].

3.5.1 Flow cytometry data analysis

After saving the data the measured values can then be illustrated in form of histogram or different dot plots. A histogram is able to display a single parameter against the number of events. A dot plot supplies a two-parameter display of data. Each dot represents a single cell that passed through the detector. There are two axes, SSC on the x-axis and FSC on the y-axis. The dispersal of the dots can differentiate cell types from each other and gate around one particular population of cells to be analyzed for their fluorescence intensity.

Gates were made by visual screening around the main population of single cells on SSC-A vs. FSC-A and FSC-H vs. FSC-A dot plots. As shown in Figure 6., normal size cells have been gated by their forward and side scatter to identify specific cell populations. By drawing a gate, we included only single cells, whereas debris and dead cell were excluded. Accordingly, we obtained living cells by using the fluorescent stain 4′,6-diamidin-2-phenylindole (DAPI). Subsequently, the fluorescence intensity of the gated cell population was determined.

The selected population of cells in the gates was analyzed for mitoxantrone fluorescence. The geometric mean of the gated fluorescence values was used for the evaluation. (Figure 6.)

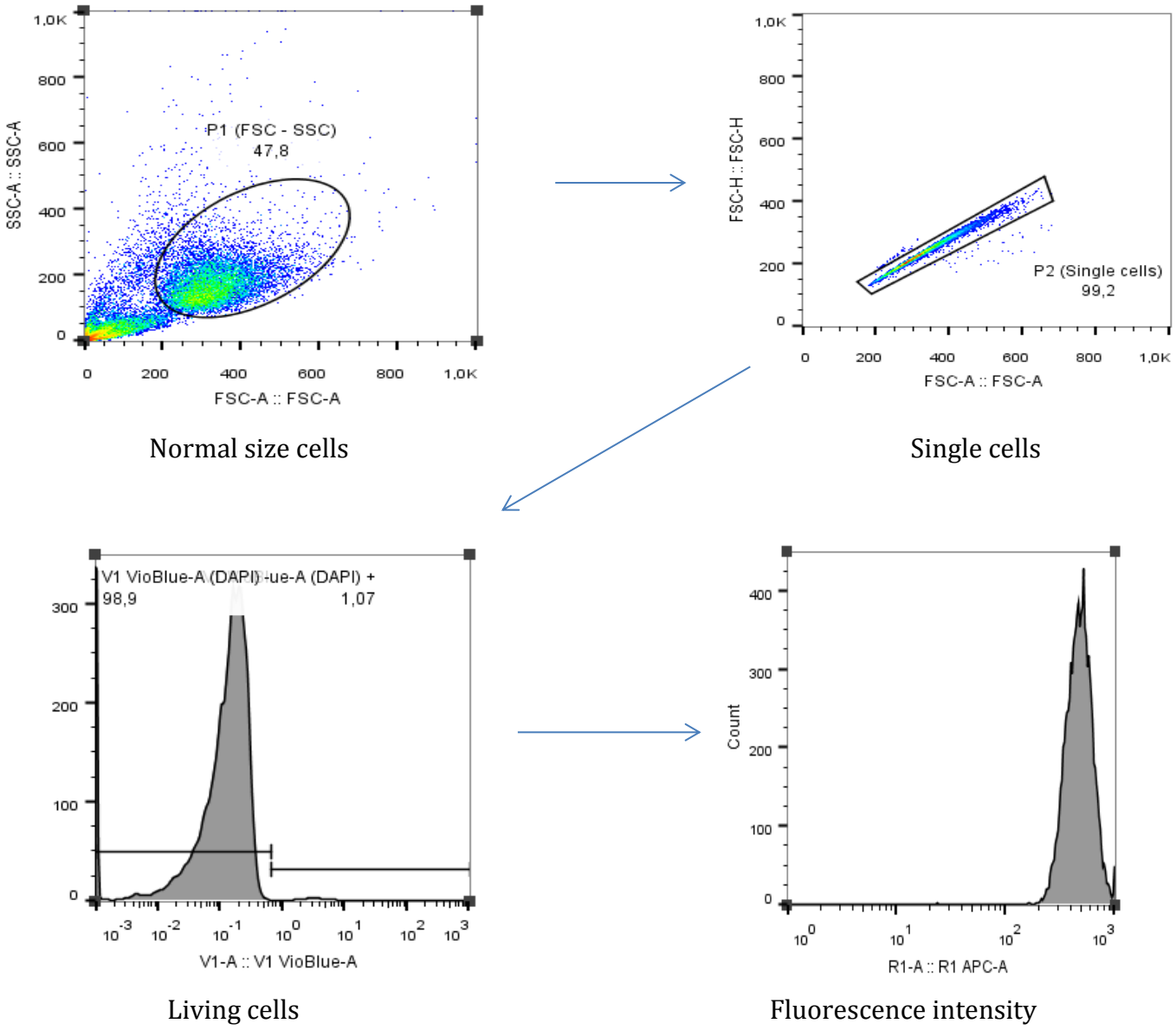


Figure6.

Flow cytometry analysis. Data presented in dot plots and histograms; each dot on the plot show an individual particle. A gate is applied to identify specific populations. Single cells were gated based on forward and side scatter, living cell detected using a stain. Fluorescence intensity (x-axis) is plotted versus the number of cells (y-axis).

3.6 Mitoxantrone accumulation assay

The synthesized test compounds were investigated for their BCRP inhibition ability by measuring the accumulation of mitoxantrone (Figure 7.).

PLB985 parental and PLB985 wtABCG2 cell lines were used in this study. Mitoxantrone is a synthetic cytostatic, immunomodulatory agent. It was selected as a fluorescent ABC transporter substrate and the synthesized compounds as an inhibitor of BCRP. For the experiment a serial dilution of the compounds in DMSO was prepared. Ko143, a selective and potent inhibitor of BCRP was used at a final concentration of 1µM as a positive control on functions of BCRP. The background fluorescence of the cell suspension was assessed in presence of 1.5% DMSO. Each sample was measured in triplicates.

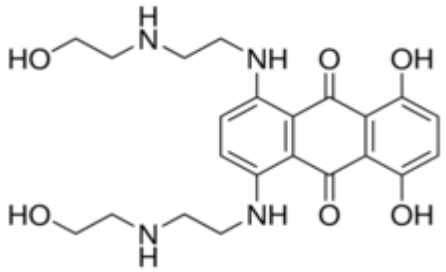


Figure7. Structure of Mitoxantrone

First of all, the used cells in culture flask (T75) were cultured and if applicable sub-cultured after reaching a cell density over 1.5*10^6/ml. Subsequently, the cell number in the resulting suspension was determined using MACSQuant flow cytometer. The needed amount of cells was transferred into a 15ml or 50ml falcon tube and centrifuged at 300 x g for 5 minutes at RT. After discarding supernatant, the cells were resuspended in 1ml HPMI (10 mM Hepes, 120 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 0.04 mM $CaCl_2$, 10 mM glucose, 10 mM $NaHCO_3$, 5 mM Na_2HPO_4, pH 7.4 with NaOH). After centrifugation again the cells were diluted to a concentration of 12×10^6 cells mL^{-1} in HPMI.

25µl of the cell suspension was added per tube, and was then pre-incubated for 5 min at 37 °C with 2 % DMSO (25 µl) in HPMI alone as a solvent control or with the 2x concentrated solutions of inhibitors in HPMI, containing 2% DMSO. Afterwards, the cells were incubated with 50 µl of a 14µM mitoxantrone stock in DMSO for 20 min at 37 °C, resulting at a 7µM final mitoxantrone and 1% DMSO concentration.

Subsequently, in order to end the accumulation of the fluorescent substrate, cells were placed in ice for 5 minutes, followed by an addition of 400µl ice cold PBS. Thereafter, the were centrifuged at 300 × g for 5 minutes at 4°C, the supernatant was removed and the cell pellet was re-suspended in 150µl cold PBS. The cells were kept on ice until measurement. Before the measurement by flow cytometer (MACSQuant, MiltenyiBiotec GmbH, BergischGladbach, Germany) 50 µl of fluorescent stain DAPI solution (4 $\mu g\, mL^{-1}$ in PBS) was added to gate out dead cells.

3.6.1 Data analysis

The mitoxantrone fluorescence intensity in every sample was corrected by the background fluorescence of the cells. Data were then normalized to the positive control Ko143, which was defined as 100%. The geometric mean of the fluorescence values was calculated for each concentration then copied to Microsoft Excel program. From the corrected data, a concentration response curve was generated by nonlinear regression using GraphPad Software (San Diego, CA) Prism version 6.05.

3.6.2 IC50 value measurements and calculations

The test compounds which have an inhibitory effect of 50% inhibition in contrast to the positive control Ko143 at a concentration of 100µM, the IC50apparent values were evaluated by measuring the accumulation in the presence of at least eight different concentration. The data are presented as mean of IC50 values ± standard deviation (SD) obtained from four or five independent experiments for each tested compound.

The assay IC50 values were calculated by non-linear regression analysis using GraphPad Prism 6, using the following equation:

$$Y = \text{Bottom} + \frac{\text{Top - Bottom}}{1 + 10((\log IC50_{apparent} - \log X) * \text{Hillslope})}$$

X: concentration of compound, Y: response, in the fluorescence intensity units
Bottom: minimum baseline response of the nonlinear fit curve, Top: maximum response
Hill-slope: steepness of the curve, IC50: concentration of compound to reach 50% of the maximum response

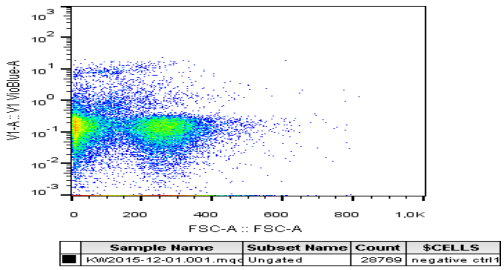
4. Results

4.1 Testing the effect of DMSO on cell viability

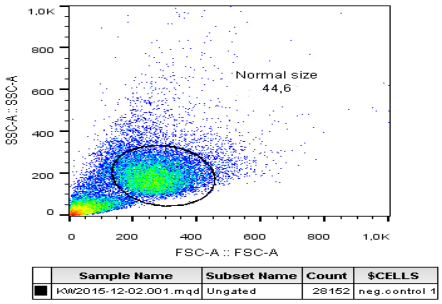
The effect of dimethyl-sulfoxide (DMSO) in different concentrations 1%, 1.5%, 3% was investigated on the cell viability. The aim of the experiment was to see if DMSO concentrations used in mitoxantrone accumulation assay have an effect on the PLB985 cell viability. The stably expressing PLB985 BCRP cells were treated with various concentrations of DMSO. DMSO is an organic solvent which also dissolves nonpolar compounds and is miscible with various organic liquids. It is used for cell freezing in biological assay as a protective agent; it prevents ice crystal formation during the procedure. Concentrated DMSO has a cytotoxic effect, higher concentrations over 10% have a harmful effect on skin and respiratory tract. First of all, we prepared the DMSO solutions in HPMI, this step includes first pipetting 25µl cells into the FACS tube, add 25µl (2% DMSO in HPMI) to simulate adding test compound, then it followed the pre-incubation in water bath at 37°C for 5 minutes, after that added 50µl of different concentrations of DMSO to simulate adding fluorescent substrate (mitoxantrone) and subsequently the incubation in water bath at 37°C for 20 minutes. The same procedure as for mitoxantrone accumulation assay was followed, as described in the materials and methods part.

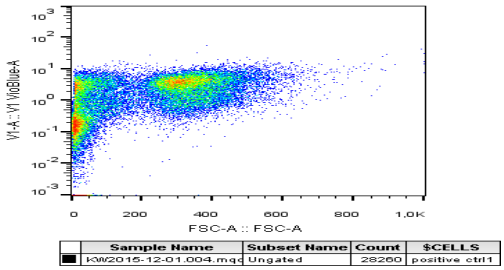
For a positive control 25µl of the cell suspension was transferred into 3 1.5 ml microcentrifuge tubes, 75µl HPMI was added and the samples were heated in Eppendorf Thermomixer at 60°C for 20 min. The heat shock was stopped by putting the microcentrifuge tubes on ice. The flow cytometer MACSQuant was used for inspection of cell viability.

After measuring the effect of 1%, 1.5%, and 3% DMSO, it was apparent that these concentrations did not have an effect on the cell viability and therefore, using any of them could not influence the outcome in the mitoxantrone accumulation assay.

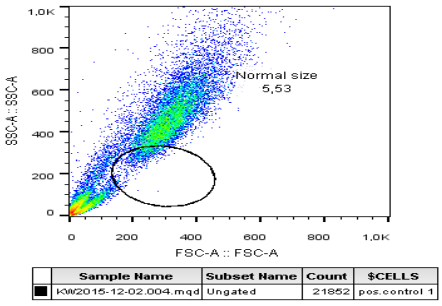


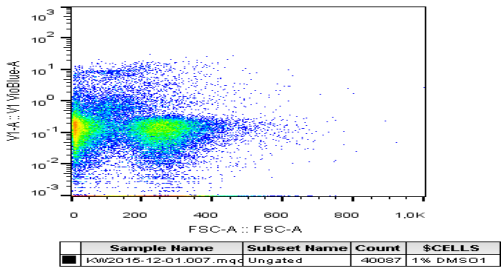
Negative control without DMSO



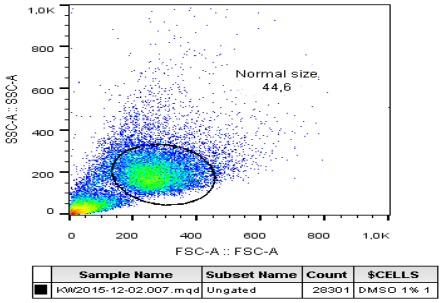


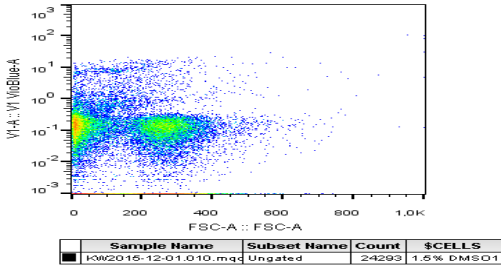
Positive control-heat shock 60°C



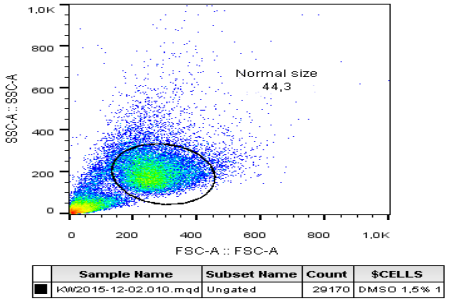


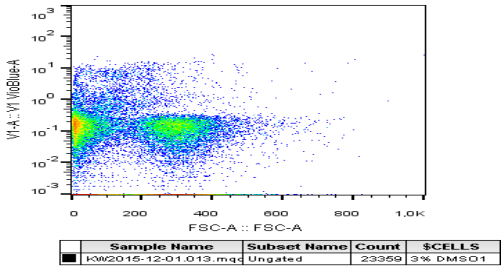
1% DMSO





1.5% DMSO





3% DMSO

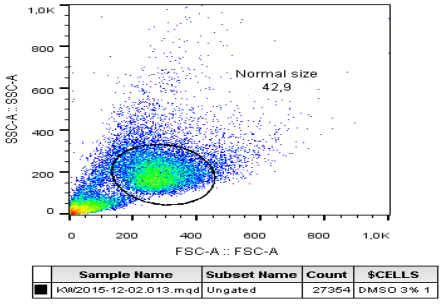


Figure8.

Fluorescence measurements of DMSO in 1%, 1.5%, 3% by flow cytometry. Dot plots of cell population obtained from SSC-A vs. FSC-A and V1-A vs. FSC-A.

4.2 Mitoxantrone accumulation experiments

4.2.1 Preliminary measurements

We performed a preliminary test of the synthesized compounds with five concentrations (0.1-500μM) to find out if they have an inhibitory potential on BCRP. Each experiment with every testing compound was repeated three times in triplicates on different day. We determined that the inhibition of all compounds at the concentration 100μM was higher than 50% and this was our threshold of considering a compound to be active.

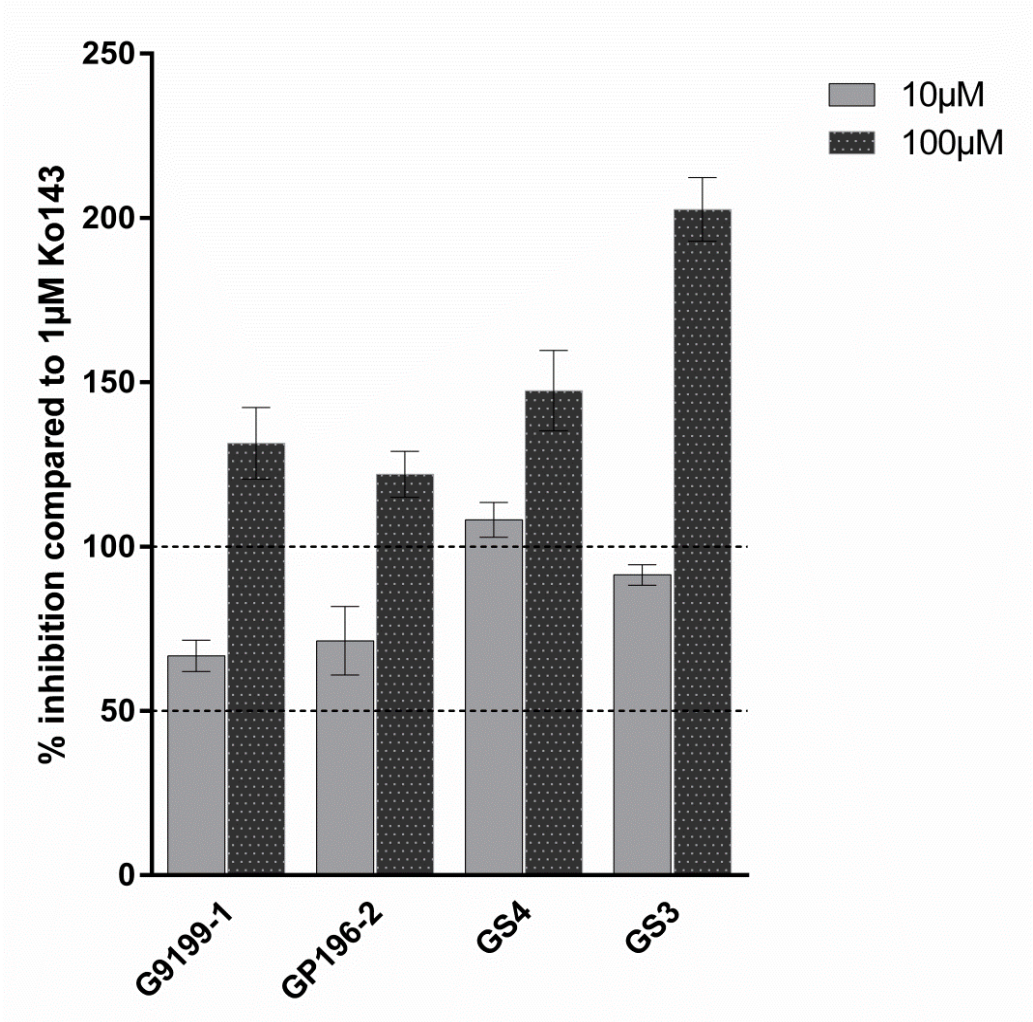


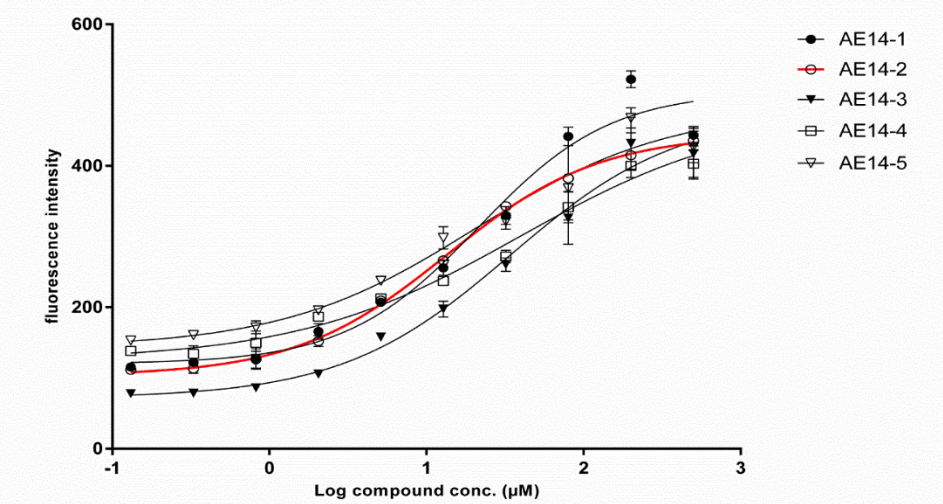
Figure9.

Effect of testing compounds on accumulation of mitoxantrone in BCRP overexpressing cells. At 100μM the inhibition of all compounds was higher than 50% → IC50 measurement.

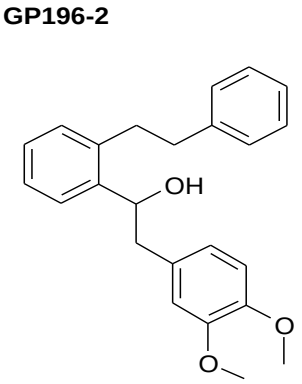
4.2.2 IC50 value measurements

The experiment for GP196-2 was performed five times with triplicate samples and the average of the IC50 value was determined. The second experiment was not included in the calculation because of a mistake in the preparation of dilutions during the experiment. The compound GP196-2 was shown to be a weaker inhibitor for BCRP (IC50: 7.2 ± 3.3 µM) (Figure 10.) compared to GS4 and GP199-1. (see Figure 14. and Table 4.)

A)



B)



C)

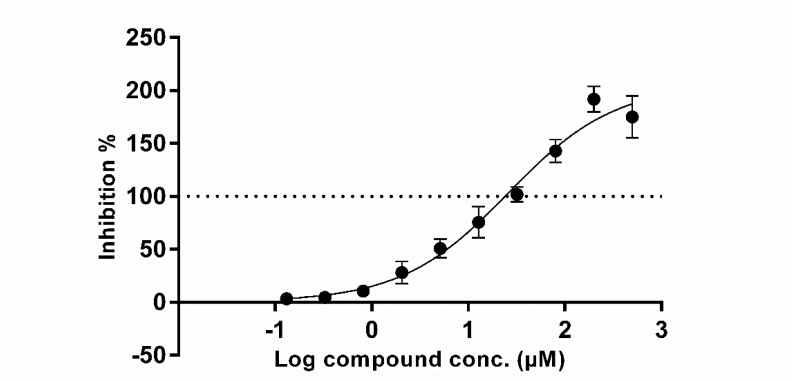
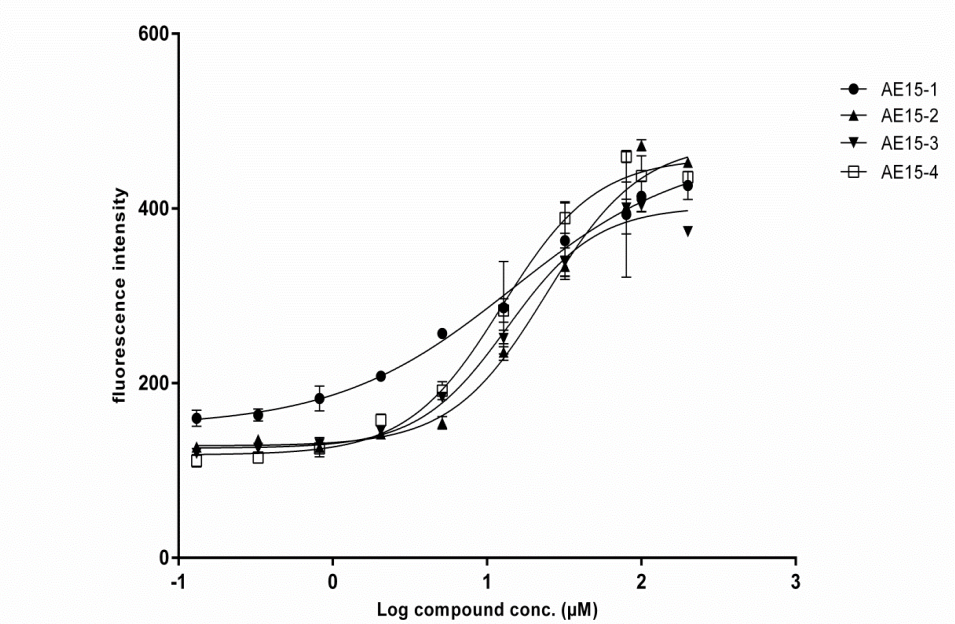


Figure10.

IC50 measurements for the compound GP196-2. A) Individual dose response curves, showing the fluorescence intensity of the accumulated mitoxantrone at different compound concentrations. The curve shown red represents an experiment which was excluded because of mistake in the procedure of the experiment. B) Structure of GP196-2. C) Average of normalized IC50 curves for the compound GP196-2. All data presented here represent the mean fluorescence intensity in percentage by subtracting the fluorescence background of the cells, afterward normalized to the positive control Ko143, set as 100%. Each data point shown represents the average of 4 independent experiments.

The experiment for GS4 was performed four times with triplicate samples and the average of the IC50 value was calculated. The compound GP196-2 was shown to be a stronger inhibitor for BCRP (IC50: 4.6 ± 1.3 μM). (Figure 11.)

A)



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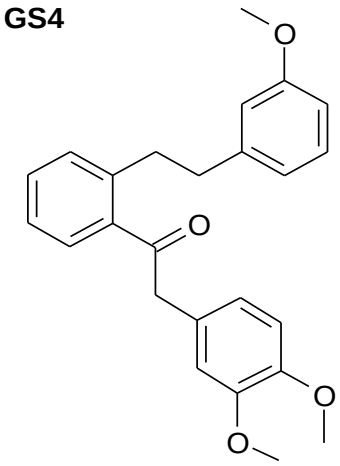


C)

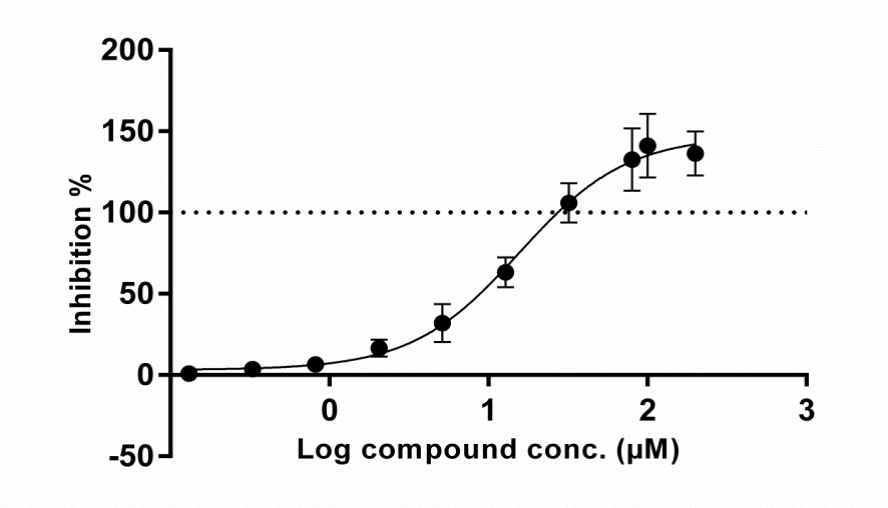
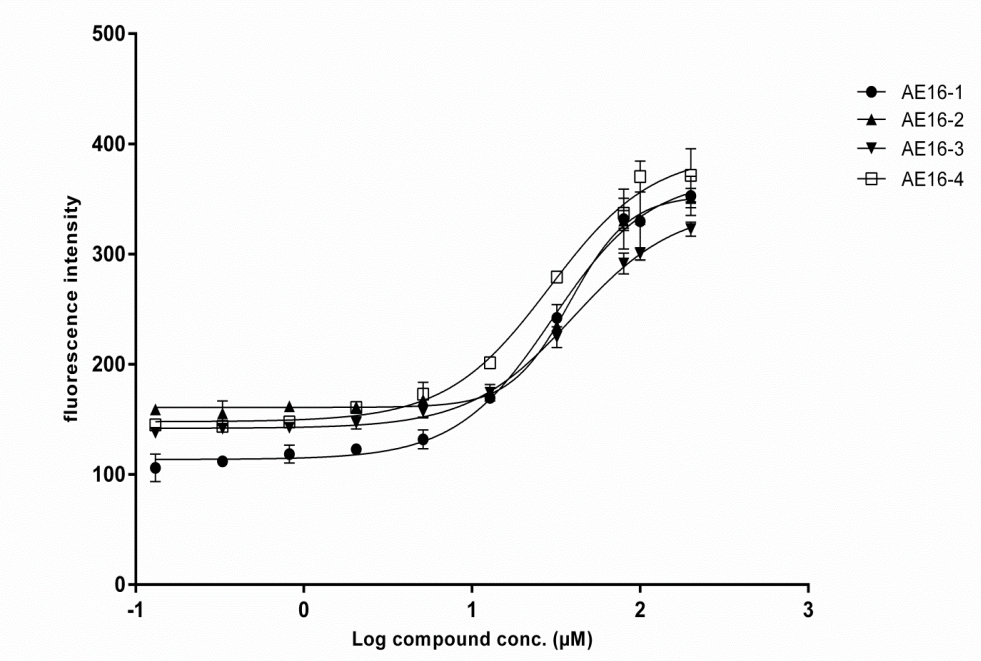


Figure11.

IC50 measurements for the compound GS4. A) Individual dose response curves, showing the fluorescence intensity of the accumulated mitoxantrone at different compound concentrations.
B) Structure of GS4. C) Average of normalized IC50 curves for the compound. Each data point shown represents the average of 4 independent experiments.

The experiment for the compound GS3 was done four times in triplicates and the average of the IC50 value was calculated. The compound GS3 revealed to be the weakest inhibitor for BCRP among the investigated compounds (IC50: 13.5 ± 4.0 μM). (Figure 12.)

A)



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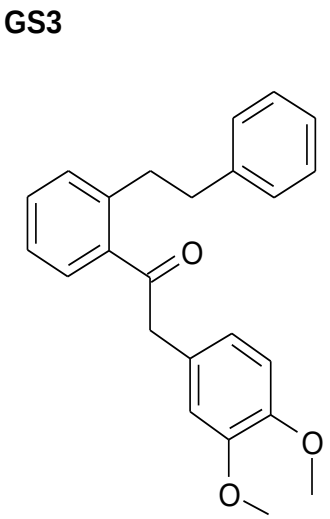

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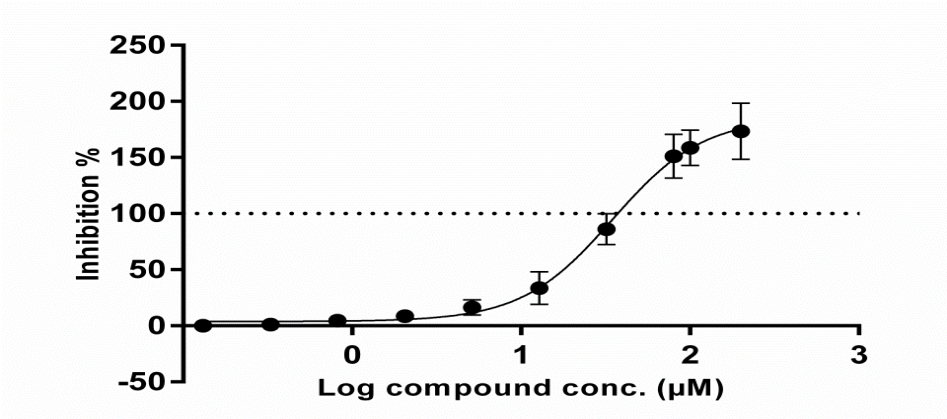
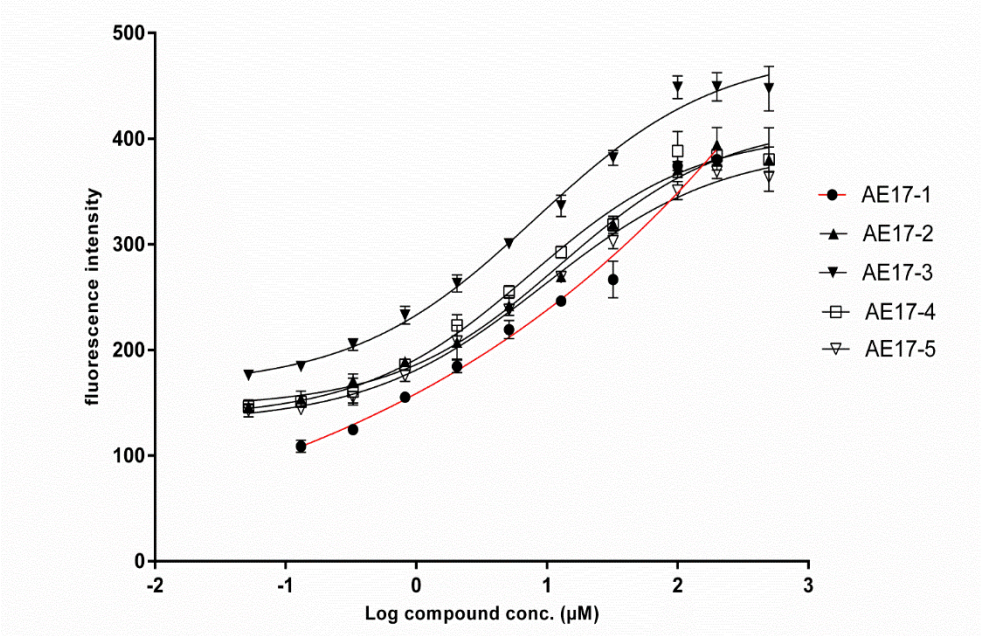


Figure12.

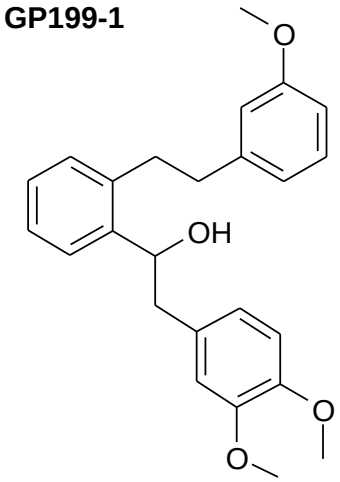
IC50 measurements for the compound GS3. A) Individual dose response curves, showing the fluorescence intensity of the accumulated mitoxantrone at different compound concentrations.
B) Structure of GS3. C) Average of normalized IC50 curves for the compound. Each data point shown represents the average of 4 independent experiments.

The experiment for GP199-1 was performed five times with triplicate samples and the average of the IC50 value was calculated. The first experiment was not included in the calculation because we used only ten different concentrations in the assay in contrast to the other experiments where twelve concentrations have been used. The compound GP199-1 showed the highest inhibitory effect for BCRP (IC50: 3.15 ± 0.7 μM). (Figure 13.)

A)



B)



C)

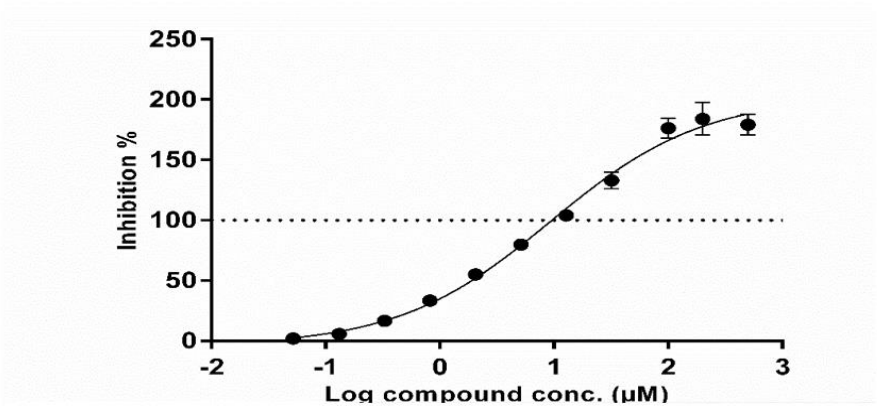
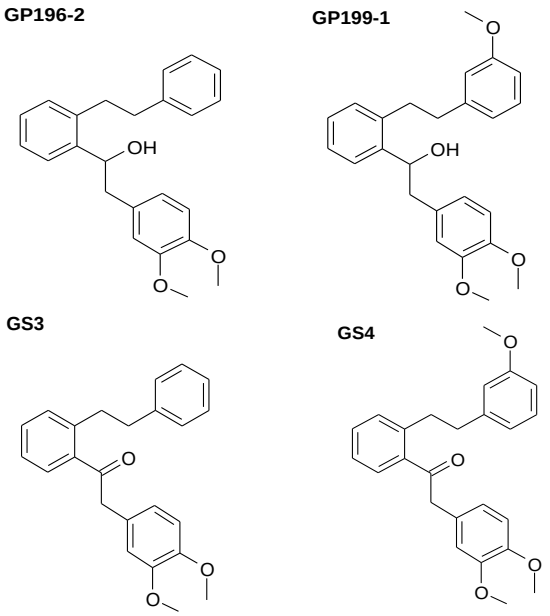


Figure13.

IC50 measurements for the compound GP199-1. A) Individual dose response curves, showing the fluorescence intensity of the accumulated mitoxantrone at different compound concentrations.
B) Structure of GP199-1. C) Average of normalized IC50 curves for the compound. Each data point shown represents the average of 4 independent experiments.

A)



B)

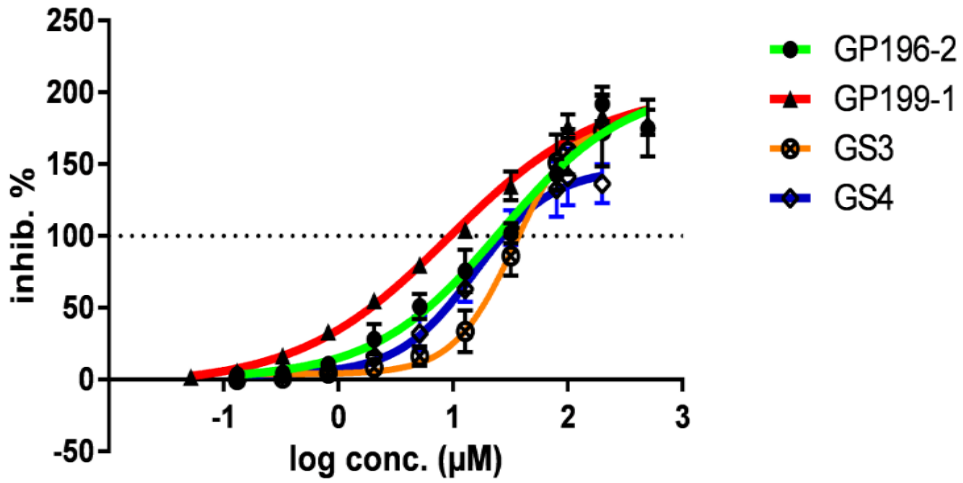


Figure18.

Representative concentration-response curves demonstrating the inhibitory activity of testing compounds. All four compounds show an inhibitory effect to BCRP, whereby GP199-1 revealed to be the most effective and potent inhibitor of BCRP.

Table 4. Summary of results

Compound	IC50 App (µM)	IC50 (µM)	Hill Slope
GP196-2	33.0 ± 15	7.2 ± 3.3	0.765 ± 0.2
GP199-1	9.19 ± 1.9	3.15 ± 0.7	0.6 ± 0.04
GS3	34.2 ± 5.5	13.5 ± 4.0	1.70 ± 0.5
GS4	16.2 ± 5.3	4.6 ± 1.3	1.27 ± 0.34

5. Discussion

BCRP was recognized lately as an efflux transporter with several major anticancer agents amongst its substrates. It plays a central role in the development of multi drug resistance in clinical medicine. There is a significant relation between BCRP overexpression and development of drug resistance in certain tumor cells, such as breast cancer, colon carcinoma and hepatocellular carcinoma. Due the high expression of BCRP in the pharmacologic barriers it affects the pharmacokinetics of drugs, which represents an obstacle in the late stages of drug development.

In our study, the intracellular accumulation level of the fluorescent BCRP substrate mitoxantrone was measured in wild-type BCRP overexpressing PLB985 cells based by flow cytometry to evaluate the BCRP inhibitory effect by the four newly synthesized compounds. The inhibitory potential of aryl-methoxy derivatives on BCRP was investigated. The presented results here show that all of these four investigated compounds have an inhibitory potential on BCRP. In the present study, we determined the IC50 value for GP199-1, GP196-2, GS4 and GS3, based on their inhibitory activity on BCRP.

The compound GP199-1 was found to be the most potent BCRP inhibitor with an IC50 of 3.20μM amongst all examined aryl methoxy derivatives, while GS3 showed the lowest inhibitory potency with an IC50 of 13.5μM (Table 4.). It was interesting to observe that compounds which bear a methoxy group on the phenyl ring were more active BCRP inhibitors than the others. Moreover, the presence of a hydroxyl group beside the methoxy groups in the structure enhances the inhibitory activity of the compound, as we can see in case of compound GP199-1. Also in other studies, it was recognized that the presence of a methoxy group is of great importance for potent inhibition of BCRP, such as it is the case with Ko143, which is a potent and specific BCRP inhibitor [28]. Compounds which possess a keto-group were found to be less active than those having a hydroxyl group.

The prediction for our test compounds was that the two with the hydroxyl group should be able to form a H-bond with BCRP while the two with the keto group should not. Nonetheless, this was not the case as shown by our experiments. A possible explanation could be that the acidic moiety in Glutamate 73 in the protein is protonated and therefore,

can interact both with H-bond donors (like hydroxyl groups) or H-bond acceptors (like keto groups) (Figure4.).

It is noticeable that the tested compounds even led to higher mitoxantrone accumulation in the BCRP expressing cells than Ko143, which is known to be the most potent BCRP inhibitor. A probable reason for that could be that the features in the structures of aryl-methoxy derivatives inhibit BCRP more than Ko143 and thus cause a higher accumulation. To prove this statement, one could use the PLB 985 parental cell line.

In conclusion, the results of this study indicate that aryl-methoxy derivatives can inhibit BCRP mediated efflux and thereby increase the intracellular substrate accumulation in BCRP overexpressing cells. These compounds as BCRP inhibitors are very promising for the ongoing research and might represent a good tool to investigate the mechanism of BCRP inhibition. The acquired knowledge can contribute to understand the role of BCRP in drug-drug interactions.

6. References

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