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# Characterization of 3-methoxy flavones for their interaction with ABCG2 as suggested by ATPase activity

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ABSTRACT

Breast Cancer Resistance Protein (BCRP/ABCG2) belongs to the superfamily of ATP binding cassette (ABC) transporters. Characteristic of some of these transporter proteins is the transport of a variety of structurally unrelated substances against a concentration gradient by using the energy of ATP hydrolysis. ABCG2 has been found to confer multidrug resistance (MDR) in cancer cells. Several anticancer drugs have been identified as ABCG2 substrates including mitoxantrone, etoposide and topotecan. As inhibition of the transporter is one of the strategies to overcome MDR, we have synthesized and tested several 3-methoxy flavones and investigated them for their ABCG2 inhibition. Among these, pentamethyl quercetin (compound **4**) and pentamethyl morin (compound **5**) were found to be fluorescent and hence screened for their possible transport by ABCG2 using confocal microscopy. This study showed that pentamethyl quercetin was far less accumulated in ABCG2 overexpressing MDCK BCRP cells as compared to MDCK sensitive cells, suggesting possible efflux of this compound by ABCG2. Pentamethyl morin showed no visible difference in both cell lines. Based on this observation, we studied several other fluorescent 3-methoxy flavones for their accumulation in ABCG2 overexpressing cells. To confirm the substrate or inhibitor nature of the tested compounds, these compounds were further investigated by ATPase assay. If stimulation of the transporter ATPase activity is detected, one can conclude that the compound is probably a transported substrate. All compounds except pentamethyl morin (compound **5**) and tetramethyl quercetin (compound **6**) were found to stimulate ATPase activity pointing to possible substrates despite being potent inhibitors of ABCG2.



## 1. Introduction

Flavonoids are a group of naturally occurring low molecular weight polyphenolic compounds that recently has been the subject of considerable scientific and therapeutic interest. They are found in a wide variety of plants and are present in fruits, roots, stems and flowers. Several beneficial effects of flavonoids on health are known and flavonoids are consumed in daily diets and in form of tea and wine [1,2]. Different flavonoids have been found to be useful as anti-inflammatory, anticancer, antidepressant, antiallergic, antiproliferative and antiangiogenic agents. Also generally a lack of toxicity is associated with this class of compounds [3]. Recently, flavonoids have received great attention for their inhibitory effect against ATP binding cassette (ABC) transporters; hence we synthesized several flavonoid analogs as ABCG2 inhibitors and investigated them extensively.

Breast cancer resistance protein (BCRP/ABCG2) is the second member of ABC transporter subfamily G and has been found to play an important role in conferring multidrug resistance in tumor cells and modulating the pharmacokinetic properties of drugs which are its substrates. It is a half transporter with a molecular weight of 72 kDa [4]. It has been proposed that, to function as an active transporter ABCG2 needs to be at least homo- or heterodimerized or higher oligomerizations are necessary [5]. Until now, several substrates including anticancer drugs such as mitoxantrone [6], topotecan [7], irinotecan [8], etc. have been identified. A few flavonoids like flavopiridol [9] and a sulfated conjugate of biochanin A [10] have also been shown to be substrates of ABCG2. In the search for potent and selective inhibitors of ABCG2, several efforts have been taken in the last decade. Recently identified inhibitors of ABCG2 belong to different chemical classes such as chalcones, chromones, flavonoids, quinazolines etc. [11–14] Several naturally occurring flavonoids as well as few synthetic analogs have been reported to be good inhibitors of ABCG2 [14–20].

Recently we studied several flavones and benzoflavones with the aim of finding new potent, selective and non-toxic inhibitors of ABCG2 [21]. In that study synthesis and biological investigation of these compounds for their inhibitory effects against major ABC transporters ABCG2, P-gp and MRP1 was reported. Among these compounds, it was observed that flavones bearing a methoxy group at position 3 of the flavone moiety showed fluorescence at 460 nm when excited at a wavelength of 355 nm. Fluorescence spectra of compounds **1**–**7** and

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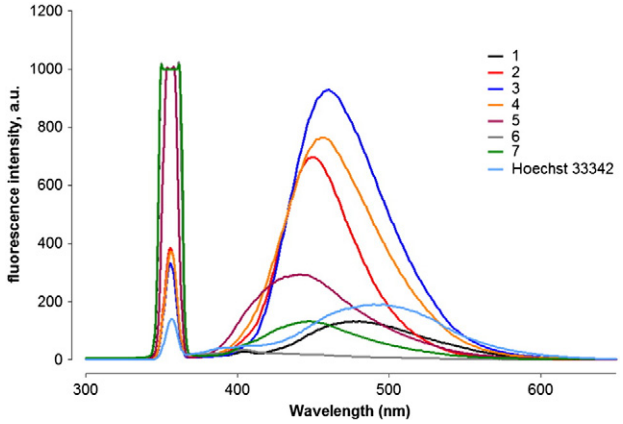
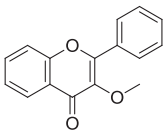
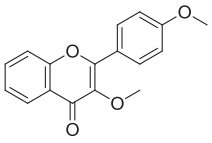
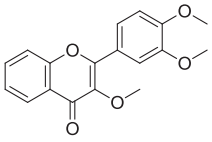
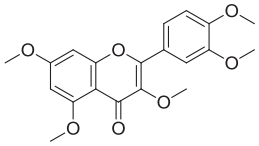
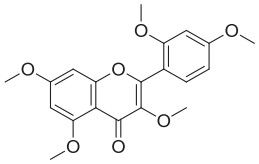
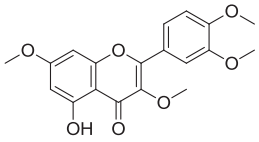
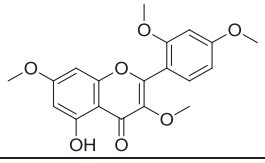



**Fig. 1.** Fluorescence spectra of 3-methoxyflavones 1–7 and Hoechst 33342.

Hoechst 33342 are shown in Fig. 1. Compound **4** (pentamethyl quercetin) and **5** (pentamethyl morin) were first investigated for their accumulation in ABCG2 overexpressing MDCK BCRP cells after 4 h incubation by confocal microscopy. Accumulation of these compounds was compared to that in sensitive MDCK cells. After comparison of fluorescence of accumulated compounds in cells using confocal microscopy, it was observed that compound **4** showed lower accumulation in MDCK BCRP cells when compared to its accumulation in non ABCG2 overexpressing sensitive cells. Such effect was not observed for compound **5**, suggesting that compound **4** could be a substrate of ABCG2 leading to its efflux and thus lower accumulation. Based on these preliminary results, other 3-methoxy flavones were then investigated for their accumulation in ABCG2 overexpressing cells by measuring their fluorescence using a microplate reader. Most of the compounds showed lower accumulation in ABCG2 overexpressing cells and to further confirm this effect, we carried out ATPase studies to test the substrate nature of selected 3-methoxy-flavones. The ATPase assay can be used to investigate interaction of compounds with ABC transporters such as ABCG2 [22]. Structures of these compounds and their inhibitory potencies against ABCG2 obtained in Hoechst 33342 assay are given in Table 1. Selected compounds were additionally investigated for their cytotoxicity and were found to have low toxicity even in the higher micro-molar range. These compounds were also investigated to test their ability to reverse the drug resistance for anticancer/cytotoxic compounds. In summary we were able to find few very potent, selective and low toxic inhibitors of ABCG2.

**Table 1**
Structures and inhibitory potencies of selected 3-methoxy flavones against ABCG2 obtained in the Hoechst 33342 and pheophorbide A assays.

| Compound | Structure | $IC_{50}$ (μM, Hoechst 33342 assay) | $IC_{50}$ (μM, pheophorbide A assay) |
|---|---|---|---|
| 1 |  | 1.21 ± 0.12 | 1.28 ± 0.17 |
| 2 |  | 7.74 ± 0.35 | 6.50 ± 0.58 |
| 3 |  | 1.18 ± 0.29 | 1.34 ± 0.19 |
| 4 |  | 0.82 ± 0.17 | 1.88 ± 0.24 |
| 5 |  | 5.98 ± 0.45 | 5.94 ± 0.74 |
| 6 |  | 0.54 ± 0.08 | 0.570 ± 0.093 |
| 7 |  | 3.27 ± 0.15 | 3.89 ± 0.16 |

## 2. Materials and methods

### 2.1. Accumulation studies of compounds **4** and **5** by confocal microscopy

Confocal microscopy was used to investigate the accumulation of fluorescent compounds **4** and **5** in ABCG2 overexpressing MDCK BCRP cells. For sample preparation, first cells were harvested after reaching a confluence of 80–90% by gentle trypsination (0.05% trypsin/0.02% EDTA) and then transferred to a 50 ml tube followed by centrifugation (266 ×*g*, 4 °C, 4 min). The cell pellet obtained was resuspended in fresh culture medium and the cell density was determined using a CASY1 model TT cell counter device (Schaerfe System GmbH, Reutlingen, Germany). Followed by another centrifugation, cells were washed three times with KHB and seeded into 6 well tissue culture plates at a density of approximately 200,000 cells per well in a volume of 2900 μl. Cells were incubated overnight and on the next day medium was changed, and 100 μl of various test compounds was added to a total volume of 3000 μl. Then the cells were further incubated for 4 h, after which the medium was removed and cells were washed two times with 1 ml PBS. After removing PBS, 1 ml of 3.7% formaldehyde in PBS solution was added and the plate was kept aside for 5 min with intermittent shaking. In the end, formaldehyde solution was removed and coverslips were fixed on the glass slide using fluomount and dried for 24 h at room temperature. For visualizing accumulated compounds in cell samples, DAPI filter was used on a Nikon A1 confocal microscope system. The results obtained for compounds **4** and **5** are depicted in Fig. 2.

### 2.2. Accumulation measurement using a microplate reader

To investigate the accumulation of compounds in MDCK BCRP cells and sensitive cells by measuring their fluorescence, cells were first prepared as described above. Cells were seeded into 96 well tissue culture plates at a density of approximately 10,000 cells per well in a volume of 100 μl culture medium. Cells were incubated overnight, on next day medium was changed and 100 μl of various test compounds were added. All test compounds had a final concentration of 25 μM. Cells were incubated with test compounds for 4 h, followed by measurement of fluorescence at an excitation wavelength of 355 nm and emission wavelength of 460 nm. As the fluorescence wavelengths of Hoechst 33342 overlap to that of compounds (excitation $\lambda = 355$ nm and emission $\lambda = 460$ nm) there was interference with the Hoechst 33342 fluorescence. As Hoechst 33342 shows almost no fluorescence in the absence of cells, blank readings were taken using only buffer solution of the same volume along with Hoechst 33342 and test compounds. This gave the fluorescence of test compounds for each concentration. This fluorescence was then deducted from the fluorescence obtained for the with cells group. This allowed us to eliminate any fluorescence interference of these compounds. Additionally the fitted top values (corresponding to maximum fluorescence at full inhibition) were compared to those obtained with a non-fluorescent standard inhibitor (XR 9577, Ko143) to ensure consistency of the subtraction procedure. This problem was not observed in the case of pheophorbide A assay as these compounds do not show any fluorescence in excitation/emission wavelengths range used for pheophorbide A assay. For normalization of protein content in each well, BCA protein assay was performed with the same 96 well plate and all fluorescence data were normalized to μg of protein. The differences in fluorescence intensities between resistant and sensitive cells were analyzed using t-test of significance. Data are shown in Fig. 3.

### 2.3. Protein expression in insect cells

High Five™ insect cells were seeded in culture flasks with Insect-XPRESS media and cultured at 27 °C. After a few passages, cells were counted by CASY1 Model TT and 18.6 Mio cells per T175 flask were

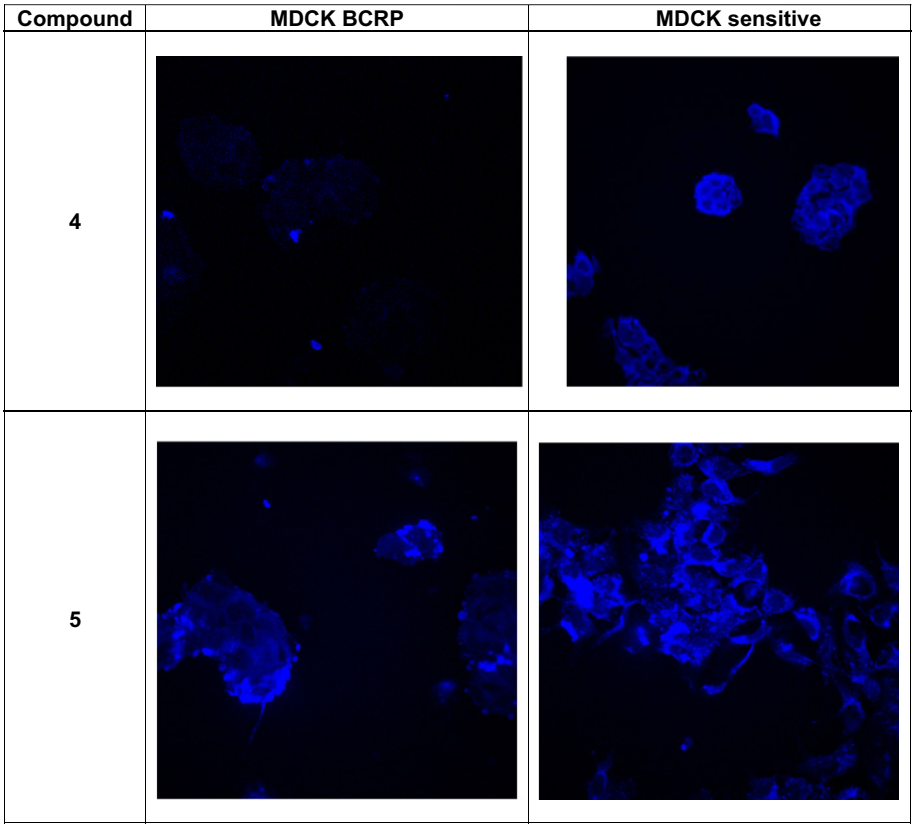



**Fig. 2.** Accumulation of flavones **4** and **5** in MDCK BCRP and MDCK sensitive cells observed by confocal microscopy.

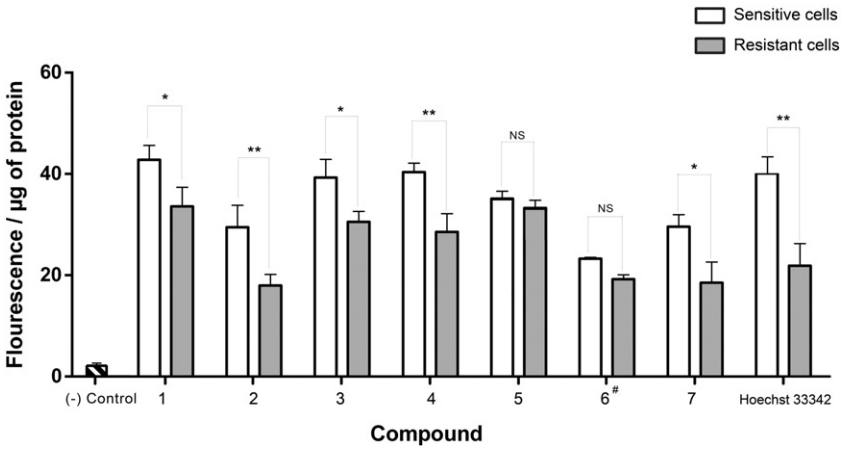



**Fig. 3.** Measurement of accumulation of 3-methoxyflavones. The fluorescence of accumulated compounds in MDCK BCRP and MDCK sensitive cells was measured using a microplate reader and the data were normalized to the protein content in each well as calculated by BCA protein estimation assay. Cells without compound were used as negative control. Data is expressed as mean ± SD of three independent experiments (NS: not significant, *$p < 0.05$, **$p < 0.01$, t-test of significance). # Compound 6 showed very low fluorescence.

seeded for baculovirus infection. Cells were infected by an Autographa californica multicapsid nuclear polyhedrosis virus (AcMNPV). 90 h after infection, when cells were swollen, they were harvested. Cell pellets were centrifuged and afterwards membrane preparation was performed. 2 ml of membrane preparation homogenization buffer (50 mM Tris pH 7.5, 2 mM EGTA pH 7.0, 50 mM Mannitol, 2 mM DTT, 1 mM PMSF, 2 μM pepstatin, 1 μM leupeptin, 1 mM benzamidine) was used for 20 million cells. Cells were disrupted by a dounce homogenizer. Cellular debris was pelleted by centrifugation at 500 ×*g* for 10 min at 4 °C and was discarded. The supernatant was ultracentrifuged at 300,000 ×*g* for 30 min at 4 °C to obtain a pellet of enriched plasma membranes. Finally membranes were resuspended in 100 μl resuspension buffer (50 mM Tris pH 7.5, 1 mM EGTA at pH 7.0, 10% (v/v) glycerol, 0.3 M mannitol, 1 mM DTT, 1 mM PMSF, 2 μM pepstatin, 1 μM leupeptin, 1 mM benzamidine) per flask and were frozen and stored at −80 °C. ATPase activity of ABCG2 in membranes of High Five insect cells was measured by a colorimetric ascorbic acid ammonia molybdate reaction described below.

### 2.4. SDS gel-electrophoresis and western blot analysis

After membrane preparation of High Five insect cells, it was necessary to check the presence of ABCG2 protein in the membranes. To confirm this, we first performed an amido black protein assay to determine the content of protein present in the membrane preparation. Afterwards 10 μg total protein was loaded onto each lane of a 4–15% Criterion TGX gel from BioRad, separated by electrophoresis and then electrotransferred onto nitrocellulose membranes. Positive control of ABCG2 as well as negative control of High Five insect cell membrane preparations not infected with ABCG2 baculovirus were also run for verification.

The nitrocellulose membranes were blocked in 5% nonfat dry milk dissolved in TBS-T (Tris-buffered saline with tween 20). For determining ABCG2 expression, blots were probed with the monoclonal antibody BXP-21 (Abcam, Cambridge, UK), which is a specific anti-BCRP antibody. As a secondary antibody, goat anti-mouse IgG HRP conjugate (H+L)-antibody (MerckMillipore, Darmstadt, Germany) was used. The immunocomplex was visualized with Thermoscientific Pierce Chemiluminescence ECL reagent. Results obtained by western blot analysis are shown in Fig. 4.

### 2.5. ATPase activity measurements

For testing the functionality of ABC transporter protein, it is necessary to determine the ATPase activity of the membrane preparation. For this purpose, membranes were analyzed using vanadate-sensitive basal and vanadate-sensitive drug-stimulated ATP hydrolysis. 10–15 μg total membrane protein was incubated in 100 μl assay buffer (50 mM Tris pH 7.5, 2 mM EGTA pH 7.0, 5 mM sodium azide, 1 mM oubain, 2 mM DTT, 50 mM KCl, 10 mM $MgCl_2$) in the presence or absence of 300 μM vanadate. The ATPase reaction was started by the addition of 5 mM ATP and all samples were incubated for 30 min at 37 °C. 25 μM prazosin and in a few experiments 25 μM quercetin were used as standard. The reaction was stopped by the addition of 100 μl of 5% SDS after 30 min. The colorimetric detection was performed afterwards by the addition of 400 μL $P_i$ reagent (1% ammonium molybdate, 0.014% antimony potassium tartrate in 2.5 N sulfuric acid), 500 μl $H_2O$ and 200 μL 1% ascorbic acid solution [23]. All samples were incubated for 10 min at room temperature and optical density was determined at 880 nm [24]. To calculate the content of liberated $P_i$, standard curves were generated using $Na_2HPO_4$.

After ensuring that the ABCG2 protein was functionally active, it was further used to investigate ATPase activity of test compounds to determine whether they stimulate ATPase activity pointing to possible transport by ABCG2. The investigated compounds were dissolved in DMSO, ensuring that the final concentration of DMSO in the assay medium was not higher than 1%. Control experiments indicated that DMSO at this concentration had no observable effect on the ATPase activity. All assays were repeated at least three times and performed in triplicates.

### 2.6. MTT cytotoxicity assay

Intrinsic cytotoxicity of most promising inhibitory compounds **1**, **3**, **4**, and **6** was determined in MDCK BCRP and MDCK sensitive cells using the MTT cytotoxicity assay. The assay was performed as described earlier with minor modifications [21,25]. Cells were seeded into 96-well tissue culture plates (Sarstedt, Newton, USA) at a density of 2500 cells per well in a volume of 180 μl and kept under 5% $CO_2$ at 37 °C for 6 h. Attachment of cells was controlled under the microscope and 20 μl of the test compounds were added to achieve the required final concentration in a volume of 200 μl. Control experiments were performed with medium containing 10% (v/v) of DMSO (positive control) and only medium (negative control). Also, dilution solvent (medium) containing the same amount of methanol (2% in highest concentration) in each dilution was studied for its effect on cell viability. After an incubation period of 72 h, the MTT reagent was added (20 μl of a 5 mg/ml solution) to each well. Plates were further incubated for 1 h and then the assay was terminated by removing supernatants and lysing the cells with 100 μl DMSO per well. Viability of cells was determined spectrophotometrically by measuring absorbance at 570 nm and background correction at 690 nm using a Thermo Scientific Multiskan® EX microplate reader.

Cytotoxicity studies were also performed to determine the drug resistance reversal ability of test compounds **1**, **3**, **4** and **6**. Final concentrations of test compounds were selected based on the $IC_{50}$ values obtained in Hoechst 33342 assay. For compounds **1** and **3** final concentrations of 1 μM and 5 μM were investigated. For compound **4** 1 μM and 2.2 μM and for compound **6** 0.5 μM and 1.8 μM were used as it is the most potent inhibitor in the data set. For this purpose SN-38 (the active metabolite of irinotecan and a substrate of ABCG2) was selected as an anti-cancer chemotherapeutic agent, and the effect of the test compounds on the cytotoxicity of SN-38 in ABCG2 overexpressing MDCK cells was studied.

### 2.7. Inhibition kinetics

To gain more detailed information about the type of interaction of pentamethyl quercetin (compound **4**) towards Hoechst 33342 and pheophorbide A, we performed extended enzyme kinetic experiments. Within this study we used various concentrations of compound **4** as well as of Hoechst 33342 or pheophorbide A respectively. For data analysis we utilized the Lineweaver-Burk linearization technique, which enabled us to determine the type of interaction between compound **4** and Hoechst 33342 or pheophorbide A.

The Hoechst 33342 assay was performed as described previously with minor modifications [21]. The concentration of Hoechst 33342 varied in the range of 0.8 μM to 2.0 μM, whereas the concentration of compound **4** varied between 0.0562 μM and 0.562 μM. Response in absence of compound **4** was used as control. Fluorescence was measured immediately after the addition of Hoechst 33342 in constant intervals of 60 s up to 120 min at an excitation wavelength of 355 nm and an emission wavelength of 460 nm, using a 37 °C tempered BMG POLARstar microplate reader.

The pheophorbide A assay was also performed as described previously with minor modifications [21]. After adding increasing concentrations of compound **4**, pheophorbide A was added in various concentrations (0.2 μM, 0.6 μM, 1.0 μM, 1.4 μM, 1.8 μM). After 120 min of incubation with the fluorescent dye, flow cytometry detection was performed using FACS Calibur (Becton Dickinson, Heidelberg, Germany). Pheophorbide A was excited at the wavelength of 488 nm, and emission was detected in the FL3 channel (670 nm).

## 3. Results and discussion

### 3.1. Investigation of selected compounds for their accumulation in ABCG2 overexpressing cells

It was observed that most of the flavonoids bearing a 3-methoxy substituent show moderate to strong fluorescence at an excitation of 355 nm and emission at 460 nm. The fluorescence property of these compounds at the wavelength of the DAPI filter (excitation $\lambda = 358$ nm, emission $\lambda = 461$ nm) made it possible to investigate their accumulation in cells using confocal microscopy. For this purpose, we decided to investigate compounds **4** and **5** for their accumulation in ABCG2 overexpressing cells with the aid of confocal microscopy. Samples were prepared by incubating test compounds at 25 μM final concentration for 4 h. Prepared samples were visualized at DAPI filter using Nikon A1 confocal microscopy system. As it can be seen in Fig. 2, compound **4** showed decreased fluorescence in ABCG2 overexpresing MDCK BCRP cells when compared to MDCK sensitive cells. No such effect was observed for compound **5**.

To quantify the accumulation fluorescence of all the test compounds, a microplate reader was used. For this purpose, samples were prepared by seeding cells in 96-well plates and incubating them with 25 μM of test compounds for 4 h in incubator maintained at 37 °C. After washing cells several times with cold buffer, fluorescence was measured at an excitation wavelength of 355 nm and emission wavelength of 460 nm. The fluorescence data were normalized to protein content in each well to correct for any cell loss in the washing steps. Fig. 3 shows the fluorescence per μg total protein content for all test compounds. It can be seen that almost all compounds showed reduced accumulation in ABCG2 overexpressing MDCK BCRP cells when compared to the corresponding sensitive MDCK cells. Hoechst 33342 was used as positive control, which is a known ABCG2 substrate, showing fluorescence at the same excitation and emission wavelengths. As it can be seen from Fig. 3, Hoechst 33342 showed highly reduced accumulation in ABCG2 overexpressing cells. Except compound **5**, all compounds showed moderate to high reduction in accumulation in ABCG2 overexpressing cells. These results suggest that compounds **1–4** and **6–7** could be substrates of ABCG2 resulting in their efflux and hence lower accumulation. To confirm this effect, we further investigated these compounds in ATPase assay using ABCG2 membrane preparations from High Five insect cells.

### 3.2. ABCG2 membrane preparation and ATPase assay

In insect cells there is a very high overexpression of target proteins, which means a big advantage over some other protein expression systems. This system is useful for both, studies in crude membrane vesicles from infected cells and for further purification and reconstitution of active transporters and afterwards studies of transporter proteins [26]. For studying ATPase activity of test compounds, purification of transporter protein is not necessary, because there are no other ATPases in the membranes of insect cells.

In our experiments, ABCG2 overexpression was generally maximal 90 h after infection with baculovirus. Functional overexpression of active ABC transport proteins in insect cells has provided new insights into the ATPase activities of the multidrug transporter. The use of crude plasma membrane prepared from BV-ABCG2 infected High Five cells, has been demonstrated to possess BCRP-associated, substrate stimulated ATPase activity [26].

The crude membrane ATPase assay described was used by several laboratories to screen potential ABCG2 substrates, based on the relatively stringent substrate-dependence of ABCG2 ATPase activity [26]. In Fig. 5 the vanadate-sensitive ATPase activity measurements of our test compounds in comparison to the standards prazosin and quercetin are depicted. All compounds were tested at a concentration of 25 μM. As it can be seen in Fig. 5, compounds **1–4** show nearly the same effects as the standards, whereas compound **5** show very low ATPase activity

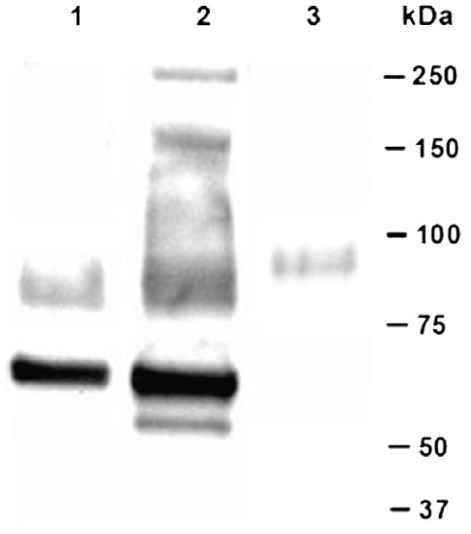



**Fig. 4.** Characterization of High Five membranes for the overexpression of ABCG2. Membrane protein preparations (10 μg) of High Five insect cells were electrophoretically separated and transferred to nitrocellulose membrane. Western blots were probed with a specific antibody against ABCG2 (BXP-21). Lane 1 shows positive control of ABCG2, lane 2 shows membrane protein preparations of High Five insect cells infected with ABCG2 baculovirus and lane 3 shows negative control of High Five insect cell membrane preparations not infected with ABCG2 baculovirus.

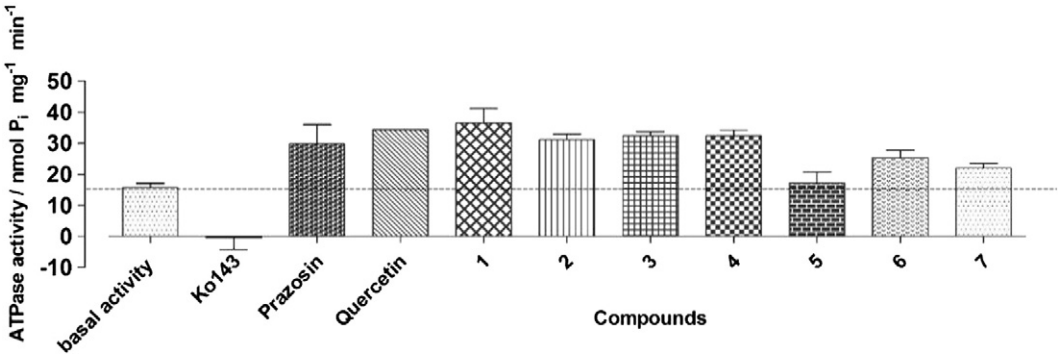



**Fig. 5.** ATPase activity of compounds 1–7 measured using High Five insect cell membranes. ATPase activity of all compounds was measured at 25 μM concentration. Prazosin and quercetin were used as positive controls, while the inhibitory effect of Ko143 was used as negative control. All data are expressed as vanadate-sensitive ATPase activity.

when compared to prazosin (standard) and only slightly higher activity than the basal activity. Compound **6** and **7** showed a moderate ATPase activity when compared to that of prazosin.

The results obtained for compounds **4** and **5** in the confocal microscopy are consistent with ATPase activity data, where the accumulation of compound **4** in ABCG2 overexpressing cells was lower than in sensitive cells, while compound **5** showed no decrease in the accumulation. From the results obtained in confocal microscopy and ATPase activity measurements, it can be suggested that compound **4** is a substrate of ABCG2. Although it is proposed to be a substrate of ABCG2, this compound was found to be also an inhibitor of ABCG2. In earlier studies we performed functional assays such as Hoechst 33342 (see Table 1) and pheophorbide A accumulation assays, in which compound **4** showed good inhibition potencies. It seems that it inhibits drug transport in a non-competitive manner towards Hoechst 33342, so it has to bind at a different binding position, see Fig. 10.

All compounds showing substantial ATPase activity were further investigated at increasing concentrations to obtain concentration response curves and the results obtained are depicted in Fig. 6. Compound **1** which has only a 3-methoxy substitution on the flavone scaffold, showed a "bell-shaped" curve which is typical for substrates having close relative affinities for two distinct binding sites [27,28]. It is assumed that the transporter contains two distinct binding sites for substrates. One of the binding sites has a high affinity towards the substrate and is activating/transporting, which is postulated to be localized in the cytoplasmic or transmembrane domains. The other one has low affinity towards the substrate and is inhibiting, this is located in the extracellular domains [16,17]. This suggests that the inhibitory effect observed at higher concentrations (second part of the curve) is believed to be due to a secondary, lower affinity binding site in the extracellular part of the transporter. If the affinities to both binding sites are close to each other, it results in a bell-shaped curve. If the affinity to the activating binding site is much higher than for the inhibitory site, a Michaelis–Menten type curve will be observed as only the activating site is targeted.

Compounds **2–4** showed typical Michaelis–Menten curves, where there was an increase in the ATPase activity with increasing compound concentration. These compounds bind at the high affinity binding site in the transmembrane or cytoplasmic regions of the transporter. Among these compounds compound **3** showed lower ATPase activity at higher concentration when compared to compounds **2** and **4**. As it can be seen by comparison of concentration response curves for compound **4** and prazosin (a well-known ABCG2 substrate), compound **4** leads to an approximately 1.5 fold higher stimulation than prazosin. The combination of compound **4** and prazosin (1:1) suggests that both bind at different binding positions because of the bell-shaped curve. In the beginning, at lower concentrations, it can be seen that binding takes place at the high affinity binding site producing an activating and at high concentrations it produces an inhibitory effect.

Compound **5** showed nearly no stimulation of ATPase activity in the ATPase assay, suggesting its non-substrate nature. This observation is in accordance with the results obtained in accumulation studies using confocal microscopy and fluorescence measurement. In these studies no substantial efflux of compound **5** was observed. Due to its very low ATPase activity, it was not possible to generate any ATPase concentration response curves.

The last two compounds **6** and **7** are different from the other investigated compounds in the respect that they bear an OH group at position 5 of the flavone scaffold. In earlier studies, presence of OH at position 5 has been shown to be important for ABCG2 inhibition. From Fig. 6 it can be seen that compound **6** shows a Michaelis–Menten inhibitory curve, while compound **7** produced a bell shaped-curve. Compound **6**, which is the most potent inhibitor in the series (see Table 1) showed very slight decrease in the accumulation in ABCG2 overexpressing cells, but due to its very low fluorescence this data is not conclusive. From the ATPase activity curve it can be seen that at low concentrations it showed rather high stimulation of ATPase activity, suggesting that it binds to the high affinity activating site of the transporter. On the other hand at higher concentrations it was able to lower the ATPase activity, suggesting that the affinities for the activating and inhibitory site are relatively close to each other. Among all tested compounds it was the most potent inhibitor, which may explain this phenomenon.

The effect of Ko143, a potent inhibitor of ABCG2, on the ATPase activity of compound **4** (25 μM) is depicted in Fig. 7. Due to inhibitory effect of Ko143 on ABCG2, it produced a Michaelis–Menten inhibitory curve. As it can be seen, higher concentrations of Ko143 were able to decrease the ATPase activity of 25 μM of compound **4**.

### 3.3. Cell viability assay

After discovering that at least one of the 3-methoxy flavones which have been shown to be inhibitors of ABCG2 in functional assays are also substrates of ABCG2, we decided to carry out in vitro cell viability assays using sensitive and ABCG2 overexpressing cells, to investigate this further. Generally, for cytotoxic compounds which are substrates of ABCG2, there is a difference in cytotoxicity in sensitive and resistant cells. Hence, if test compounds are cytotoxic and show lower cytotoxicity in resistant cells than in the sensitive cells, we could conclude that these compounds are being transported by ABCG2 leading to lesser intracellular accumulation and lower toxicity.

For this purpose, we carried out cell viability assays for compounds **1**, **3**, **4** and **6** by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. The toxicity studies of these compounds were performed using MDCK BCRP and sensitive MDCK cells. The concentration–response curves obtained in MTT assays

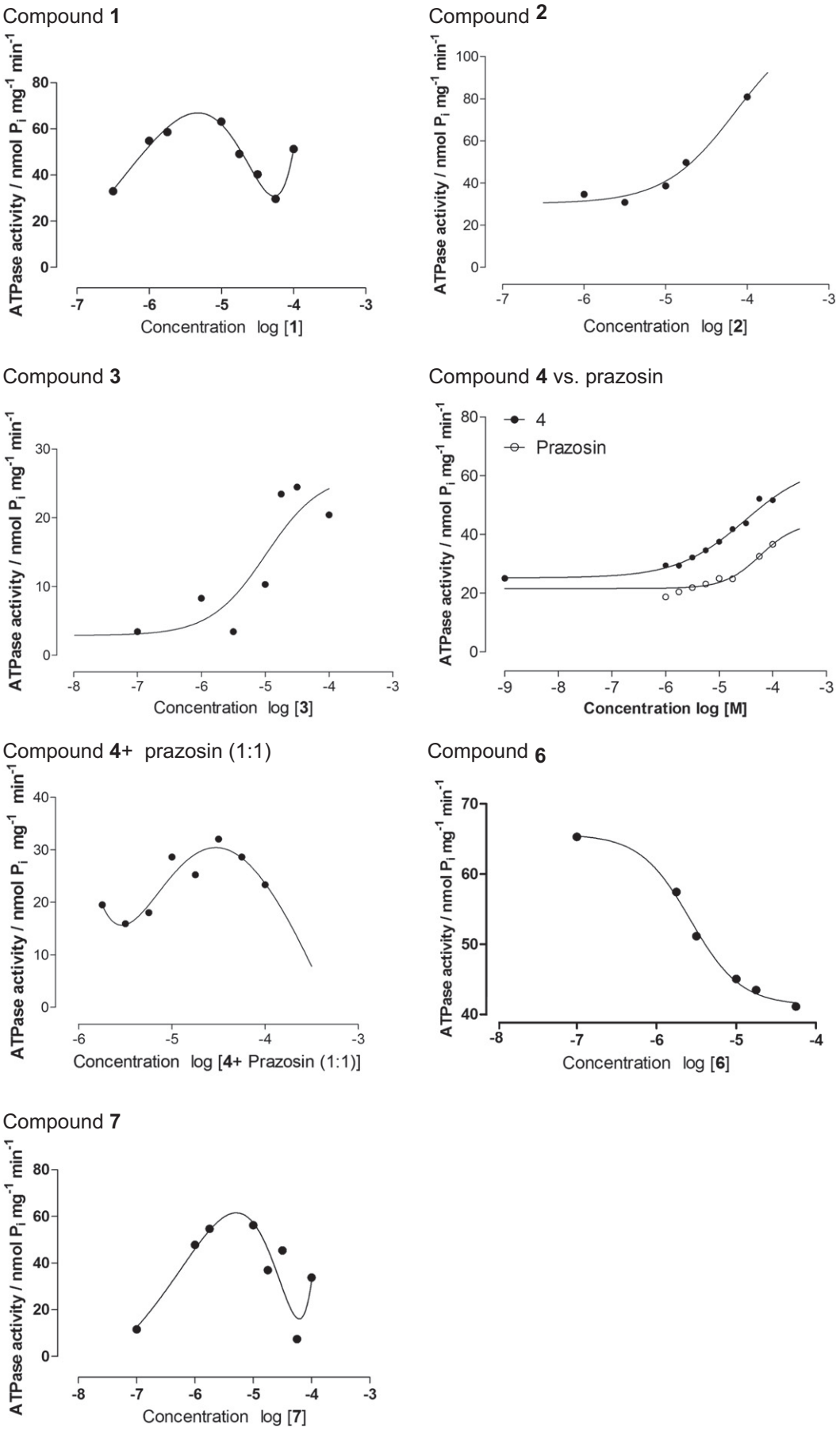



**Fig. 6.** Concentration response curves for compounds 1–7 in the ATPase assay. High Five insect cell ABCG2 membrane preparations were used for carrying out ATPase activity measurements.

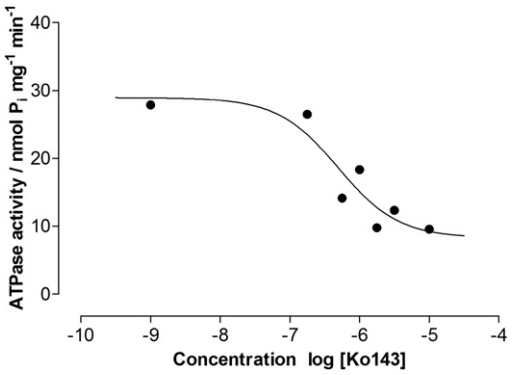



**Fig. 7.** Effect of Ko143 on ATPase activity in the presence of compound 4. Effect of increasing concentration of ABCG2 inhibitor Ko143 on the ATPase activity of compound 4 (25 μM) was studied.

for compounds **1**, **3**, **4** and **6** are shown in Fig. 8. It can be seen that all four compounds investigated showed very low cytotoxicity. These compounds showed a decrease in cell viability only at very high concentrations. Due to their low cytotoxicity a substantial difference in cytotoxicity in sensitive and resistant cells was not observed. Compound **4** that is supposed to be a substrate was even slightly more toxic against the MDCK BCRP cells than against sensitive MDCK cells (Fig. 8). Hence, it is not possible to conclude about the substrate nature of these compounds based on their cytotoxicity.

We also investigated these ABCG2 inhibitors if they have the ability to overcome drug resistance to another cytotoxic ABCG2 substrate in ABCG2 overexpressing cells. For this purpose test compounds **1**, **3**, **4** and **6**, which showed highest ABCG2 inhibition in Hoechst 33342 and pheophorbide A accumulation assays, were investigated for their ability to reverse resistance towards SN-38 in MDCK BCRP cells. Compounds **1** and **3** were investigated at final concentrations of 1 μM and 5 μM, compound **4** at final concentrations of 1 μM and 2.2 μM and compound **6** at final concentrations of 0.5 μM and 1.8 μM. Fig. 9 depicts the shift in the dose–response curves to SN-38 in MDCK BCRP cells in presence of the test compounds. This shift in dose–response curves towards lower concentrations indicates sensitization of MDCK BCRP cells to cytotoxicity of SN-38. All the compounds were able to significantly reverse the resistance even at low micromolar concentrations and a complete reversal of resistance was observed at higher concentrations selected for each compound.

### 3.4. Inhibition kinetics

To get more detailed information related to the type of interaction of selected flavonoids with ABCG2 in the presence of other substrates, we performed enzyme kinetic studies with compound **4**. This compound was found to have a substrate nature in microscopy studies as well as in ATPase activity studies. In enzyme kinetic experiments we varied both, the concentrations of Hoechst 33342 and compound **4**, as well as the concentrations of pheophorbide A and compound **4**. The enzyme kinetics data obtained allowed us to determine the type of interaction between the compound **4** and Hoechst 33342 or pheophorbide A through application of the Lineweaver–Burk linearization technique. The obtained Lineweaver–Burk plots for both kinetic binding studies are depicted in Figs. 10 and 11.

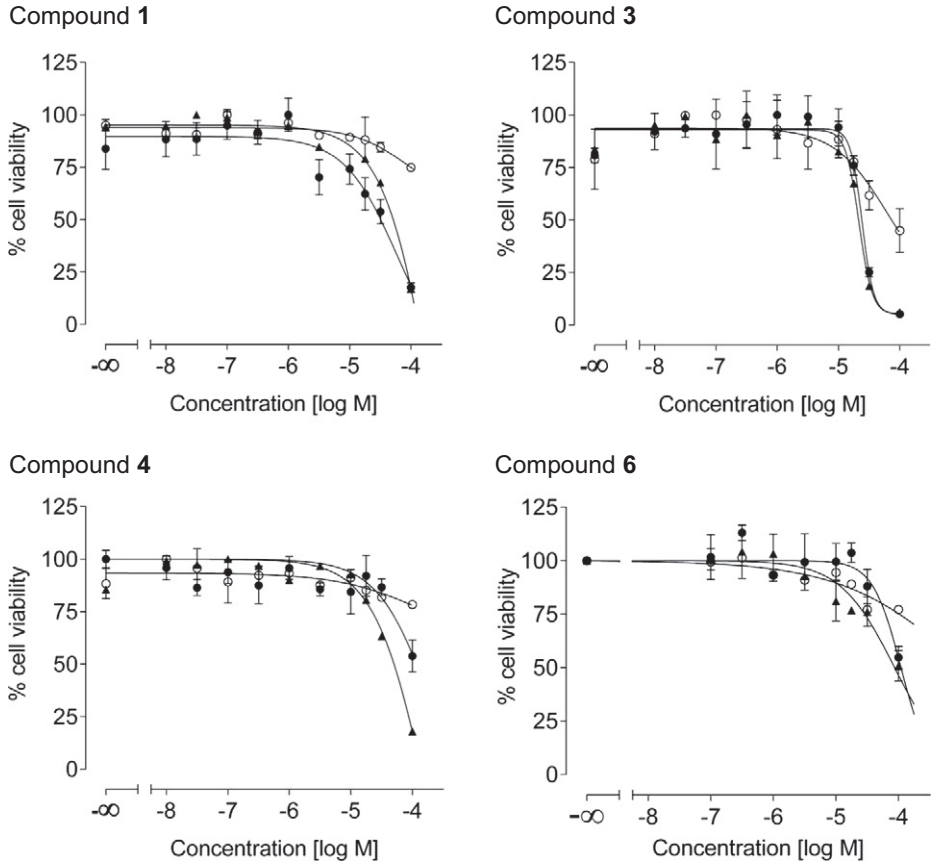



**Fig. 8.** Cytotoxicity of selected test compounds in MDCK cells. Cytotoxicity of selected test compounds was determined in the MTT assay using MDCK BCRP and sensitive MDCK cells. Compounds **1**, **3**, **4** and **6** were investigated up to 100 μM concentration for 72 h. Closed triangle: MDCK BCRP cells, closed circle: MDCK sensitive cells and open circle: dilution solvent (medium) containing 2% methanol in the highest concentration.

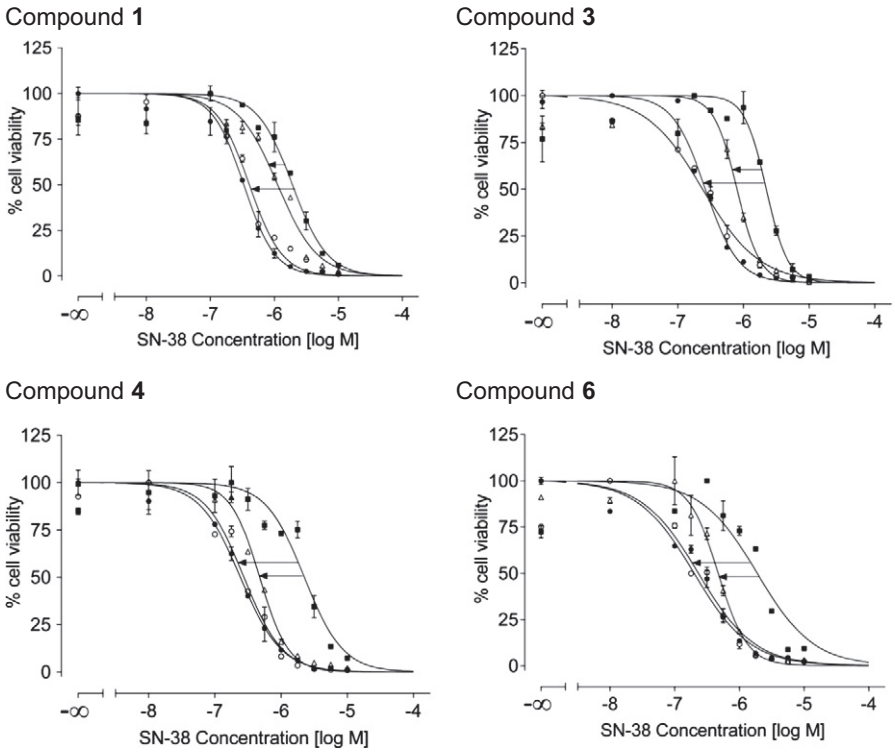



**Fig. 9.** Effect of test compounds on cytotoxicity of SN-38 in ABCG2 overexpressing cells. Dose–response curves of SN-38 cytotoxicity. The effect of compounds **1**, **3**, **4** and **6** on SN-38 cytotoxicity in MDCK cells is shown. Concentrations of test compounds were selected according to their inhibitory potencies. Compounds **1** and **3** were investigated at 1 μM (open triangles) and 5 μM (open circles) final concentrations. Compound **4** was investigated at 1 μM (open triangles) and 2.2 μM (open circles) final concentrations. Compound **6** was investigated at 0.5 μM (open triangles) and 1.8 μM (open circles) final concentrations. ABCG2 overexpressing MDCK cells without inhibitor (closed squares) showed less sensitivity towards SN-38, than sensitive MDCK cells (closed circles).

Experiments carried out with Hoechst 33342 had difficulty at higher concentrations due to overlapping fluorescence of Hoechst 33342 and compound **4**. Therefore concentrations selected for the compound **4** were restricted to a lower range where the fluorescence of the compound **4** was not dominating. Maximum inhibition was inferred from the Hoechst 33342 fluorescence in presence of the non-fluorescent ABCG2 inhibitor Ko143. From Fig. 10, it can be suggested that there is a non-competitive interaction between compound **4** and Hoechst 33342, indicating that it binds at a distinct site on ABCG2 than that of Hoechst 33342. Similar results were obtained for enzyme kinetic studies carried out using pheophorbide A, where a non-competitive interaction was also observed between pheophorbide A and compound **4**. Here, no interference between the fluorescence of the flavonoid with fluorescence of pheophorbide A was observed.

## 4. Conclusions and summary

In the current study we investigated several fluorescent 3-methoxy flavones for their substrate nature by ABCG2. These compounds were previously found to be good inhibitors of ABCG2. From the current study, we can conclude that all selected compounds except compound **5** and **6** seem to be substrates of ABCG2, although they are inhibitors of ABCG2 as identified in Hoechst 33342 and pheophorbide A accumulation assays. Compounds **1** and **7** produced bell-shaped ATPase activity curves, which is characteristic for typical membrane active transporters, whose transport site alternates between high and low affinity state [29]. Compound **6** showed activation at very low concentrations while producing inhibitory effect at higher concentrations. Compounds **2**–**4** produced

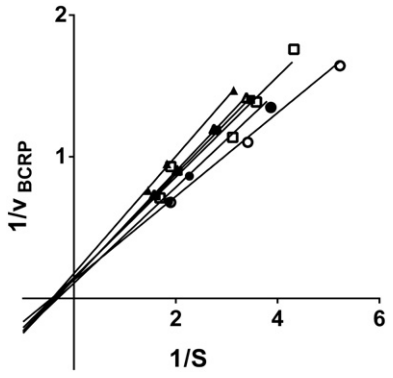



**Fig. 10.** Interaction of compound 4 with Hoechst 33342. Lineweaver–Burk plots for compound **4** showing a non-competitive interaction type with Hoechst 33342. Control (open circle), 0.0562 μM (closed circle), 0.1 μM (open square), 0.178 μM (closed square), 0.316 μM (open triangle), 0.562 μM (closed triangle).

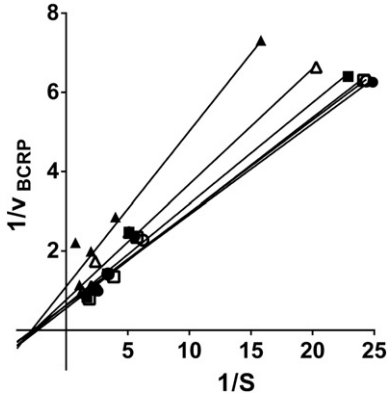



**Fig. 11.** Interaction of compound 4 with pheophorbide A. Lineweaver–Burk plots for compound **4** showing a non-competitive interaction type with pheophorbide A. Control (open circle), 0.1 μM (closed circle), 0.316 μM (open square), 0.562 μM (closed square), 1.0 μM (open triangle), 1.78 μM (closed triangle).

activating Michaelis–Menten ATPase activity curves and showed less accumulation in ABCG2 overexpressing MDCK BCRP cells in comparison to sensitive cells, which suggests that they were transported by ABCG2.

Compound **4** being fluorescent and the strongest inhibitor of ABCG2 in this examination set after compound **6** (which showed no substrate nature of ABCG2), we decided to carry out extensive studies with this pentamethoxy flavone. Lower accumulation of this compound in ABCG2 overexpressing MDCK BCRP cells when compared to its accumulation in non ABCG2 overexpressing sensitive cells was confirmed by confocal microscopy studies. In ATPase activity studies it showed an approximately 1.5 fold higher stimulation of ATPase activity in comparison to prazosin, a well-known substrate of ABCG2. The combination of compound **4** and prazosin (1:1) resulted in a bell-shaped ATPase activity curve, which suggests different binding positions of these two compounds. Interaction of compound **4** with prazosin by enzyme kinetic studies could not be verified due to experimental limitations. At lower concentrations, it can be seen that binding takes place at the high affinity binding site producing an activating and at high concentrations it produces an inhibitory effect. We were also able to show the inhibition of Ko143 on the ATPase activity of selected compound **4**.

In cell viability assay, compound **4** was found to have low toxicity even in the higher micro-molar range and was able to reverse the drug resistance for SN-38 in ABCG2 overexpressing cells. In enzyme kinetic studies of compound **4**, we were able to show a non-competitive interaction of compound **4** towards Hoechst 33342 as well as towards pheophorbide A. From these observations it can be suggested that, although 3-methoxy flavones are good inhibitors of ABCG2, they are also substrates and their inhibitory activity could be due to their fast diffusion.

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