Karanjin interferes with ABCB1, ABCC1, and ABCG2

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ABSTRACT - PURPOSE: The prominent ATP-binding cassette (ABC) transporters ABCB1, ABCC1, and ABCG2 are involved in substance transport across physiological barriers and therefore in drug absorption, distribution, and elimination. They also mediate multi-drug resistance in cancer cells. Different flavonoids are known to interfere with different ABC transporters. Here, the effect of the furanoflavonol karanjin, a potential drug with antiglycaemic, gastroprotective, antifungal, and antibacterial effects, was investigated on ABCB1, ABCC1, and ABCG2-mediated drug transport in comparison to the flavonoids apigenin, genistein, and naringenin. METHODS: Cells expressing the relevant transporters (ABCB1: UKF-NB-3^{ABCB1}, UKF-NB-3^{r}VCR^{10}; ABCC1: G62, PC-3^{r}VCR^{20}; ABCG2: UKF-NB-3^{ABCG2}) were used in combination with specific fluorescent and cytotoxic ABC transporter substrates and ABC transporter inhibitors to study ABC transporter function. Moreover, the effects of the investigated flavonoids were determined on the ABC transporter ATPase activities. RESULTS: Karanjin interfered with drug efflux mediated by ABCB1, ABCC1, and ABCG2 and enhanced the ATPase activity of all three transporters. Moreover, karanjin exerted more pronounced effects than the control flavonoids apigenin, genistein, and naringenin on all three transporters. Most notably, karanjin interfered with ABCB1 at low concentrations being about 1µM. CONCLUSIONS: Taken together, these findings should be taken into account during further consideration of karanjin as a potential drug for different therapeutic indications. The effects on ABCB1, ABCC1, and ABCG2 may affect the pharmacokinetics of co-administered drugs.

INTRODUCTION

ATP binding cassette (ABC) transporters are ATP-dependent pumps that transport substances across biological membranes. They play important roles in the passage of drugs, xenobiotics, and food constituents through cellular and tissue barriers and consequently in their absorption, distribution, and excretion. Moreover, different ABC transporters are frequently found highly expressed on cancer cells playing an important role in cancer cell chemoresistance (1-4).

Flavonoids are plant polyphenols known to be present in tea, vegetables, nuts, citrus fruits, and herbal products. They belong to the most frequently consumed natural products. Many flavonoids have been found to interfere with various ABC transporters (5,6). These effects are of interest for two different reasons: 1) flavonoids may interfere with the absorption, distribution, and excretion of drugs or xenobiotics through their effects on ABC transporters 2) a number of different flavonoids is under investigation as anti-cancer agents and effects on ABC transporters are relevant for their anti-cancer activities, especially in combination with other anti-cancer drugs that interfere with ABC transporters and/or are ABC transporter substrates (5,6). Moreover, many flavonoids are discussed as chemopreventive agents (7,8).

Karanjin is a flavonoid isolated from karanja (*Pongamia glabra* Vent.) and other Pongamia species used as bio-insecticide and bio-pesticide (9-12). Moreover, karanjin has been reported to exert antiglycemic, gastroprotective, antifungal, and antibacterial effects (11-17) and to be very well tolerated in rats (17).

Here, we demonstrate that karanjin interferes with the action of the ABC transporters ABCB1 (also known as P-glycoprotein, the gene is also known as multi-drug resistance gene 1, *MDR1*), ABCC1 (also known as multi-drug resistance-associated protein 1, MRP1), and ABCG2 (also known as breast cancer resistance protein 1, BCRP1).

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This represents the first investigation of the effects of the drug candidate karanjin on these three major ABC transporters. Moreover, we show that karanjin exerts substantially enhanced effects on ABCB1 compared to apigenin, genistein, and narigenin, three flavonoids that were used as control compounds because they are known to interfere with ABC transporters.

MATERIALS AND METHODS

Drugs

Karanjin was obtained from Indofine Chemical Company Inc. (Hillsborough, NJ, USA) via ABCR GmbH & Co. KG (Karlsruhe, Germany). Vincristine, verapamil, PGP-4008, NEM (N-ethylmaleimide), and GS (reduced glutathione) were obtained from Sigma-Aldrich (Deisenhofen, Germany). Rhodamine 123, MK571, sulfasalazine, and 5-CFDA (5-Carboxyfluorescein diacetate) were purchased from Merck Biosciences (Darmstadt, Germany). Mitoxantrone was received from GRY Pharma (Kirchzarten, Germany). Ko 143 was purchased from TOCRIS Bioscence (Bristol, UK). BODIPY-FL-prazosine (BODIPY-prazosine) was obtained from Invitrogen GmbH (Frankfurt/Main, Germany).

Cell lines

The neuroblastoma cell line UKF-NB-3 was established from bone marrow metastasis of a stage IV neuroblastoma patient with MYCN-amplification (18). ABCB1 -overexpressing (UKF-NB-3^{ABCB1}, control cell line UKF-NB-3^{Cer2}) or ABCG2-overexpressing (UKF-NB-3^{ABCG2}, control cell line UKF-NB-3^{iG2}) sub-lines of UKF-NB-3 were established by lentiviral transduction with LeGO vectors encoding ABCB1, ABCG2, or corresponding control vectors, respectively. Vectors were derived using standard molecular cloning techniques as described before (19,20) (see also www.lentigo-vectors.de). UKF-NB-$3^{r}VCR^{10}$ is a UKF-NB-3 sub-line characterised by high ABCB1 expression that was established by adaptation of UKF-NB-3 to growth in the presence of vincristine 10ng/ml (18,21).

The glioblastoma cell line G62 (22) was obtained from Dr. Dorothee von Laer (Medical University, Innsbruck, Austria). Among ABC transporters investigated, it shows expression of ABCC1 and ABCG2 (Suppl. Figure 1). Using the ABCC1-specific fluorescence substrate 5-CFDA or vincristine, a cytotoxic drug transported by ABCC1 but not by ABCG2, G62 represents a suited model to study ABCC1 function. The MABCC1-specific ABC transporter inhibitor MK571 but not the ABCG2-specific inhibitor Ko143 or the ABCB1-specific inhibitor PGP-4008 caused 5-CFDA accumulation in G62 cells and sensitised G62 cells to vincristine cytotoxicity (Suppl. Figure 1).

All cell lines were propagated in IMDM supplemented with 10 % FBS, 100 IU/ml penicillin and 100 mg/ml streptomycin at 37°C. Cells were routinely tested for mycoplasma contamination.

Viability assay

Cell viability was tested by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay after 120 h incubation, modified as described before (21). All experiments were performed at least in triplicate.

Flow cytometry and investigation of efflux of ABC transporter substrates

Antibodies directed against ABCB1 (Alexis Biochemicals via AXXORA Deutschland, Lörrach, Germany), ABCC1, or ABCG2 (both Kamiya Biomedical Company, Seattle, WA, USA), followed by secondary antibody labelled with Phycoerythrin (R&D, Wiesbaden, Germany) were used to detect protein expression by flow cytometry (FACSCalibur, BD Biosciences, Heidelberg, Germany).

ABC transporter-mediated drug efflux and wash-out kinetics were determined as described before (20,21). To investigate ABCB1 -mediated substance efflux, cells were preincubated with different concentrations of karanjin for 30 min. 10 µM verapamil was used as positive control for ABCB1 -mediated efflux. 0.1 µM rhodamine 123 was added for another 30 min. Then, cell culture medium was removed, cells were washed three times with PBS, and fresh medium containing drugs was added. After another 45 min, cellular fluorescence was analysed by flow cytometry. Rhodamine 123 was detected at the FL1 channel.

For wash out kinetic experiments, UKF-NB-3^{ABCB1} cells were incubated for 1h with rhodamine 123 0.1 µM together with different concentrations of karanjin or with verapamil 10 µM (positive control for that inhibits ABCB1 function). Subsequently, cells were washed, re-suspended in supplemented medium, and the cellular fluorescence was measured after different time points (t_0, t_5, t_{15}, t_{30}, t_{60}, t_{120} minutes) by flow cytometry.

To investigate ABCC1- or ABCG2-mediated efflux, the same procedures were carried out. For investigation of the influence of karanjin on ABCC1 function, G62 cells were used. 5-CFDA (detected at the FL1 channel) served as

fluorescent ABCC1 substrate and vincristine as cytotoxic ABCC1 substrate.

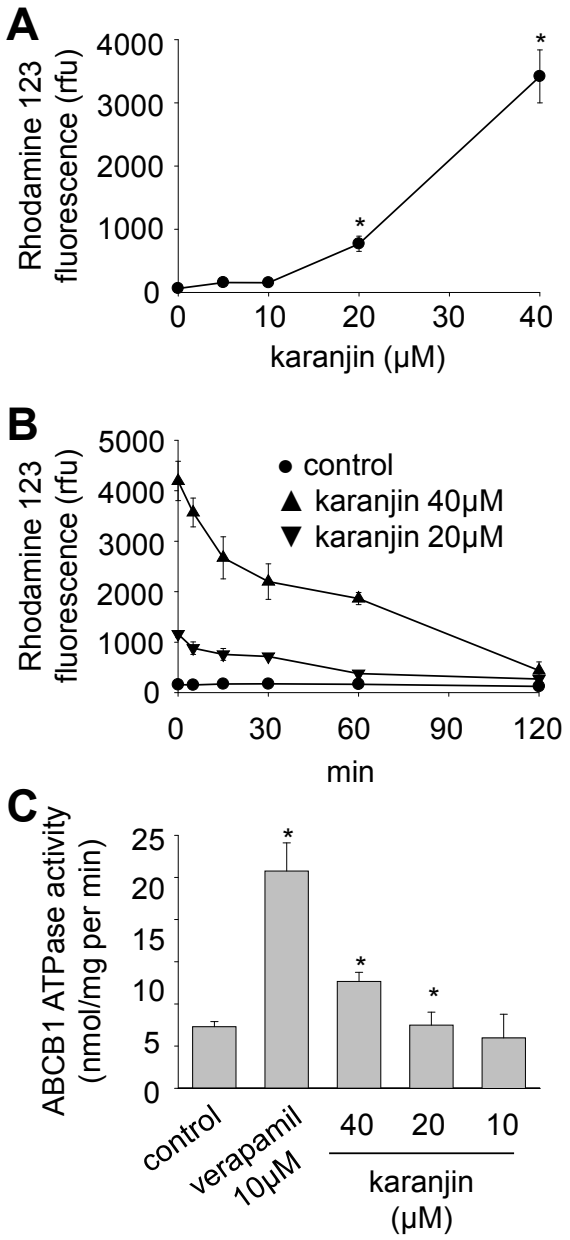

Figure 1. Interference of karanjin with P-glycoprotein (ABCB1) activity. A) Concentration-dependent influence of karanjin on rhodamine 123 (0.1 μM) fluorescence in UKF-NB-3^{ABCB1} cells, B) time kinetics of rhodamine 123 (0.1 μM) fluorescence in UKF-NB-3^{ABCB1} cells in the presence of karanjin after a 60 min pre-incubation period with subsequent wash-out of extracellular rhodamine 123 and karanjin (control = rhodamine 123 incubation in the absence of drugs). C) ABCB1 ATPase activity in isolated membranes in the presence of karanjin (control = activity in the absence of drugs). Verapamil, a known ABCB1 substrate, was used for comparison. * $P < 0.05$ relative to non-treated controls.

The ABCC1 inhibitor MK571 was used as control substance. For ABCG2, UKF-NB-3^{ABCG2} cells were used. BODIPY-prazosine served as fluorescent ABCG2 substrate (detected at the FL1 channel). Mitoxantrone served as cytotoxic ABCG2 substrate. The ABCG2 inhibitor Ko143 was used as control substance.

Determination of ATPase activity

The ATPase activities of ABCB1, ABCC1, and ABCG2 were determined using membrane preparations (ABCB1-Membran: BD Biosciences, Heidelberg, Germany; ABCC1- and ABCG2 membranes: Solvo Biotechnology, Budapest, Hungary) and an established kit (BD Biosciences, Heidelberg, Germany) following the manufacturer's instruction.

STATISTICAL ANALYSIS

Two groups were compared by t-test. More groups were compared by ANOVA with subsequent Student-Newman-Keuls test.

RESULTS

Influence of karanjin on ABCB1 activity

Karanjin concentrations ≤ 40 μM increased accumulation of the fluorescent ABCB1 substrate rhodamine 123 in ABCB1-expressing UKF-NB-3^{ABCB1} cells (but not in empty vector-transduced control UKF-NB-3^{Cer2} cells or non-transduced parental UKF-NB-3 cells that lack ABCB1 expression, not shown) in a concentration-dependent manner (Figure 1A). However, karanjin 40 μM did not affect ABCB1 expression (Suppl. Figure 2A).

Next, we investigated the ABCB1 wash-out kinetics of karanjin. Cells were incubated with rhodamine 123 in the presence of karanjin 60 min. Subsequently, cells were washed and cellular fluorescence was monitored at different time points by flow cytometry. The wash-out of karanjin resulted in a fast decrease of rhodamine 123 fluorescence (Figure 1B). Moreover, karanjin increased ABCB1 ATPase activity (Figure 1C). Next the influence of karanjin was investigated on toxicity exerted by the ABCB1 substrate vincristine in UKF-NB-3^{ABCB1} cells (Figure 2A). The effects were compared to those of apigenin, genistein, and naringenin that are flavonoids known to interfere with function of these ABC transporters (6,23-25). Karanjin 20μM caused the strongest sensitisation to vincristine resulting in an 85-fold decrease of the vincristine IC_{50} value whereas apigenin 20μM (5-fold), genistein 20μM

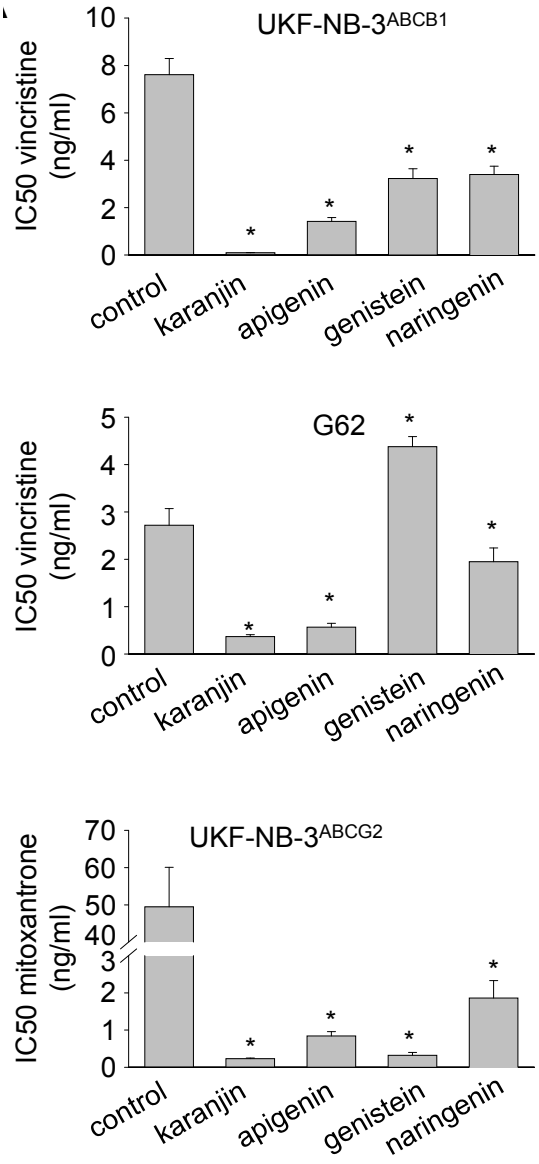

Figure 2. Influence of karanjin on cancer cell sensitivity to cytotoxic ABC transporter substrates in comparison to other flavonoids. A) Concentrations of vincristine (cytotoxic substrate of P-glycoprotein (ABCB1)) that decrease cell viability by 50% (IC50) in ABCB1-expressing UKF-NB-3^{ABCB1} cells in the presence of karanjin 20μM (cell viability in the absence of vincristine is 76.13 ± 9.52% relative to control), apigenin 20μM (73.27 ± 13.05%), genistein 20μM (32.60 ± 8.09%), or naringenin 20μM (62.68 ± 15.98%). B) Vincristine (cytotoxic substrate of ABCC1) IC50s in ABCC1-expressing G62 cells in the presence of karanjin 20μM (92.73 ± 3.94%), apigenin 20μM (82.56 ± 7.64%), genistein 20μM (79.04 ± 3.75%), or naringenin 20μM (84.13 ± 5.44%). C) Mitoxantrone (cytotoxic substrate of ABCG2) IC50s in ABCG2-expressing UKF-NB-3^{ABCG2} cells in the presence of karanjin 20μM (83.73 ± 15.22%), apigenin 20μM (77.26 ± 12.63%), genistein 20μM (62.03 ± 10.68%), or naringenin 20μM (64.82 ± 9.90%).* $P < 0.05$ relative to non-treated controls.

(2-fold), or naringenin 20μM (2-fold) exerted only moderate effects (Figure 2A). Similar relative changes in the effects on vincristine activity were detected when we used the investigated flavonoids at a concentration of 10μM (Suppl. Table 1).

Sensitisation of UKF-NB-3^{ABCB1} cells to vincristine by karanjin occured in a dose-dependent manner with karanjin 1.25 μM still resulting in a statistically significant (2.5-fold) reduction of the vincristine IC_{50} (Suppl. Table 2). Similar results were obtained in UKF-NB-3^{r}VCR^{10} cells (Suppl. Table 2), a sub-line of UKF-NB-3 adapted to growth in the presence of vincristine 10ng/ml, that is characterised by high ABCB1 expression (18,21). The vincristine IC50s of the parental cell line UKF-NB-3 and the vector control UKF-NB-3^{Cer2} were not influenced by karanjin (Suppl. Table 2).

Influence of karanjin on ABCC1 activity

Karanjin increased accumulation of the fluorescent ABCC1 substrate 5-CFDA in ABCC1-expressing G62 cells in a concentration-dependent manner (Figure 3A) but did not influence ABCC1 expression after incubation for up to 5 days (Suppl. Figure 2B).

The determination of wash-out kinetics resulted in a rapid decline of 5-CFDA fluorescence in the presence of karanjin (Figure 3B) and karanjin increased ABCC1 ATPase activity (Figure 3C).

While genistein 20μM increased the vincristine IC_{50} in G62 cells (1.6-fold), apigenin 20μM, naringenin 20μM, or karanjin 20μM reduced vincristine IC_{50}s (Figure 2B). Karanjin 20μM caused the strongest reduction (7-fold) followed by apigenin 20μM (5-fold) and naringenin 20μM (1.4-fold). Similar relative changes in the effects on vincristine activity were detected when we used the investigated flavonoids at a concentration of 10μM (Suppl. Table 1).

Moreover, karanjin sensitised G62 cells and PC-3^{r}VCR^{20} cells (another ABCC1 model, see (20,21)) to vincristine in a dose-dependent fashion (Suppl. Table 2). In both cell lines, karanjin 5μM was the lowest concentration that significantly reduced the vincristine IC_{50} (G62: 2.3-fold; PC-3^{r}VCR^{20}: 1.8-fold).

Influence of karanjin on ABCG2 activity

Treatment of ABCG2-expressing UKF-NB-3^{ABCG2} cells with karanjin in the presence of the fluorescent ABCG2 substrate BODIPY-prazosine resulted in a dose-dependent increase in cellular BODIPY-prazosine fluorescence (Figure 4A).

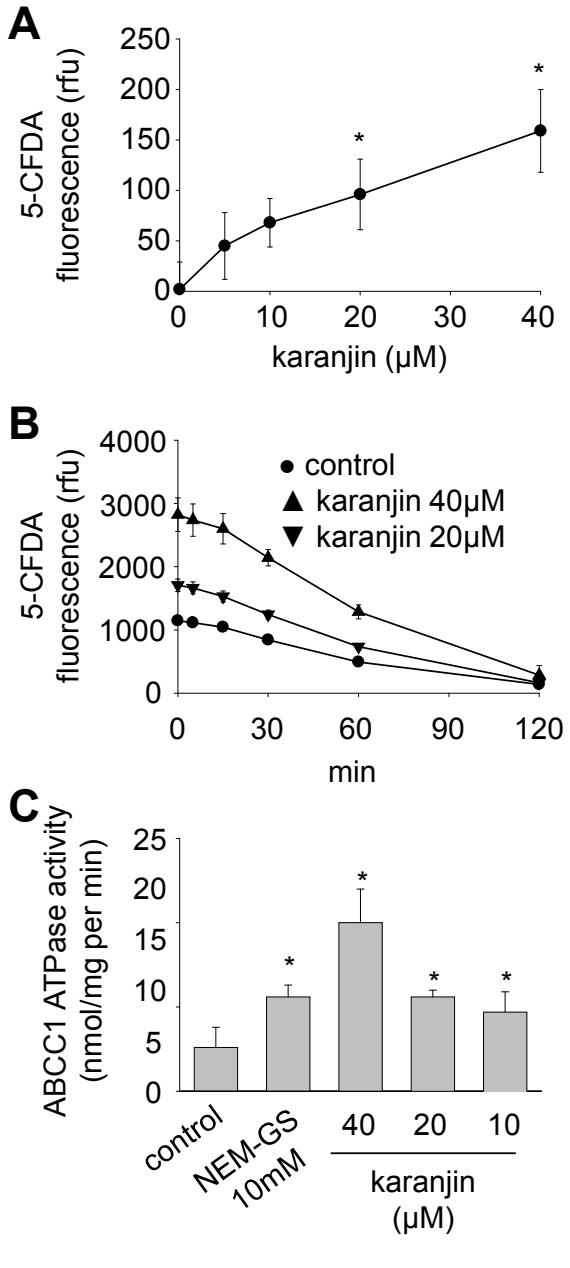

Figure 3. Interference of karanjin with ABCC1 activity. A) Concentration-dependent influence of karanjin on 5-CFDA (0.2μM) fluorescence in G62 cells, B) time kinetics of 5-CFDA (1μM) fluorescence in G62 cells in the presence of karanjin after a 60 min pre-incubation period with subsequent wash-out of extracellular 5-CFDA and karanjin (control = 5-CFDA incubation in the absence of drugs). C) ABCC1 ATPase activity in isolated membranes in the presence of karanjin (control = activity in the absence of drugs). NEM-GS (a freshly prepared 1:1 mixture of NEM and GS), a known ABCC1 substrate, was used for comparison. * $P < 0.05$ relative to non-treated controls

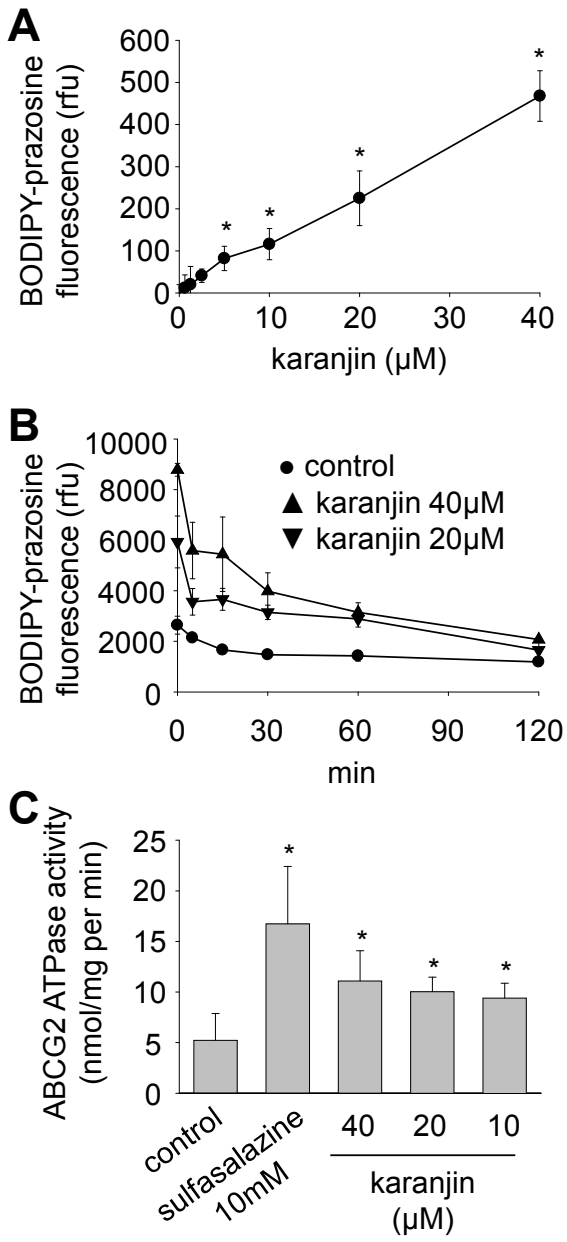

Figure 4. Interference of karanjin with ABCG2 activity. A) Concentration-dependent influence of karanjin on BODIPY-prazosine (0.2 μM) fluorescence in UKF-NB-3^{ABCG2} cells, B) time kinetics of BODIPY-prazosine (1 μM) fluorescence in UKF-NB-3^{ABCG2} cells in the presence of karanjin after a 60 min pre-incubation period with subsequent wash-out of extracellular BODIPY-prazosine and karanjin (control = BODIPY-prazosine incubation in the absence of drugs). C) ABCG2 ATPase activity in isolated membranes in the presence of karanjin (control = activity in the absence of drugs). Sulfasalazine, a known ABCG2 substrate, was used for comparison. * $P < 0.05$ relative to non-treated controls

In contrast, karanjin did not affect BODIPY-prazosine fluorescence in UKF-NB-3 cells or UKF-NB-3 cells transduced with the control vector (UKF-NB-3^{iG2}, data not shown). In addition, karanjin did not influence ABCG2 expression after incubation for 5 days (Suppl. Figure 2).

The cellular BODIPY-prazosine fluorescence levels rapidly declined after wash-out of karajin in UKF-NB-3^{ABCG2} cells (Figure 4B) and karanjin increased ABCG2 ATPase activity (Figure 4C).

Karanjin 20µM (217-fold), apigenin 20µM (59-fold), genistein (156-fold), as well as naringenin 20µM (27-fold) sensitised UKF-NB-3^{ABCG2} cells to the cytotoxix ABCG2 substrate mitoxantrone (Figure 2C). Similar relative changes in the effects on mitoxantrone activity were detected when we used the investigated flavonoids at a concentration of 10µM (Suppl. Table 1). Moreover, karanjin sensitised UKF-NB-3^{ABCG2} cells to mitoxantrone in a dose-dependent manner but did not affect mitoxantrone sensitivity of UKF-NB-3 cells or UKF-NB-3^{iG2} cells (Suppl. Table 2).

DISCUSSION

Flavonoids are ubiquitous constituents of our daily diet. Many pharmacological actions have been ascribed to this group of plant secondary metabolites (5-8). Many flavonoids are known to interfere with ABC transporter-mediated transport of drugs and xenobiotics (5,6). Here, we show that the flavonoid karanjin that has been reported to exert antiglycemic, gastroprotective, antifungal, and antibacterial effects and to be very well tolerated in rats (11-17) interferes with the cellular drug efflux through the major ABC transporters ABCB1, ABCC1, and ABCG2. This finding is of general importance for the assessment of karanjin as drug candidate for different diseases since ABC transporters play key roles during drug absorption, distribution, and excretion (2) and thus potential interactions between karanjin and other ABC transporter substrates (e.g. co-administered drugs, xenobiotics) will have to be considered.

Moreover, ABC transporter inhibitors, particularly ABCB1 inhibitors, have been intensively investigated as anti-cancer drugs (1-4,26). The use of first generation inhibitors such as verapamil or cyclosporine A (that were established drugs found to interfere with ABCB1) was limited by toxic (off-target) side effects (1-4). Later generations were more specific and acted as non-competitive ABC transporter inhibitors. Nevertheless, although some clinical phase I and II trials with ABC transporter inhibitors appeared to be encouraging, successful phase III trials are missing (1-4). Possibly, broad spectrum ABC transporter inhibitors might be needed since chemoresistant cancer (stem) cells express multiple ABC transporters and inhibition of one is not sufficient (27). Interestingly in this context, karanjin appears to exert stronger effects on ABCB1, ABCC1, as well as ABCG2 than the three flavonoids apigenin, genistein, and naringenin that are known to interfere with ABC transporter-mediated substance transport (6,23-25) and that were therefore used for comparison. Notably, genistein protected G62 cells from vincristine-induced cytotoxicity. This might be due to cytoprotective activities including antioxidative and anti-apoptotic effects that have been attributed to flavonoids like genistein (28,29). Noteworthy in this context, karanjin consistently sensitised ABC transporter-expressing tumour cells derived from three different entities (neuroblastoma, glioblastoma, prostate carcinoma) to varying anti-cancer drugs.

Particularly, the effects of karanjin on ABCB1 function were substantially higher (85-fold sensitisation of ABCB1-expressing UKF-NB-3^{ABCB1} cells to the ABCB1 substrate vincristine by karanjin 20µM) than the effects mediated by the same concentration of apigenin, genistein, or naringenin that ranged between 2- and 5-fold sensitisation. Karanjin significantly sensitised UKF-NB-3^{ABCB1} cells to vincristine (2.5-fold) in a concentration as low as 1.25µM. Docking experiments did not indicate a significant difference in the binding affinities of karanjin, apigenin, genistein, naringenin to ABCB1 (Suppl. Figure 3). This suggests that the differences detected in the effects on ABCB1 function between the investigated flavonoids may not be a consequence of differences in their binding to ABCB1. Hydrophobicity and a planar structure have been described to be important for flavonoid interaction with ABCB1 (30). Looking at the chemical structures of the investigated flavonoids (Figure 5), karanjin is the only substance lacking hydroxyl groups. Moreover, karanjin possesses in contrast to the other flavonoids a furan group (furanoflavonoid). Therefore, its pronounced effects on ABCB1 appear to be in accordance with the established structure-activity-relationships.

In conclusion, we show that the flavonoid karanjin interferes with substance transport mediated by the major ABC transporters ABCB1, ABCC1, and ABCG2. The effects exerted by karanjin appear to be more pronounced than those of apigenin, genistein, or naringenin, other

flavonoids with documented effects on ABC transporters that were used as control substances.

Most notably, karanjin interfered with ABCB1-mediated drug efflux in very low micromolar concentrations. Due to the important roles of ABC transporters in drug absorption, distribution, and excretion these findings should be taken into account during further consideration of karanjin as a drug candidate exerting antiglycemic, gastroprotective, antifungal, and antibacterial effects. Moreover, broad spectrum ABC transporter inhibitors might have a role in anti-cancer therapies since high ABC transporter expression represents a central cancer cell chemoresistance mechanism.

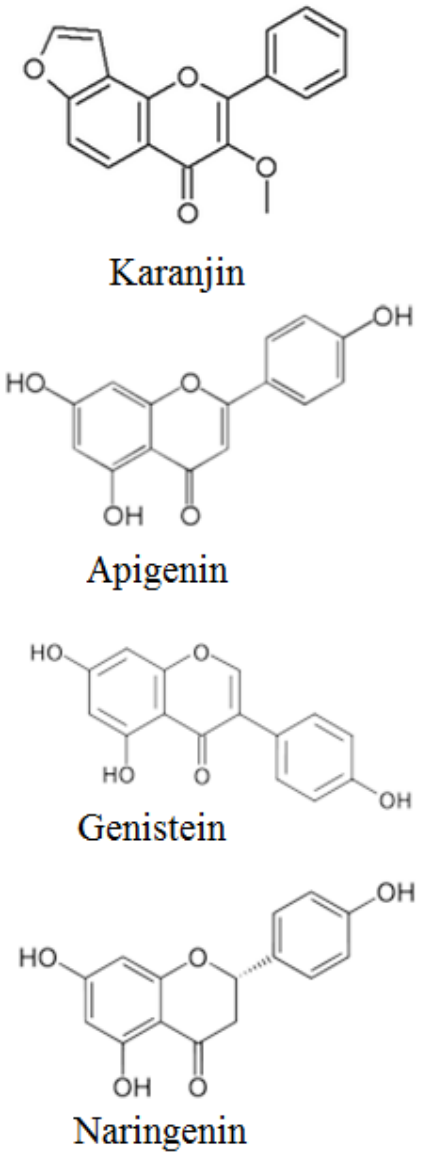

Figure 5. Chemical structures of the flavonoids karanjin, apigenin, genistein, and naringenin.

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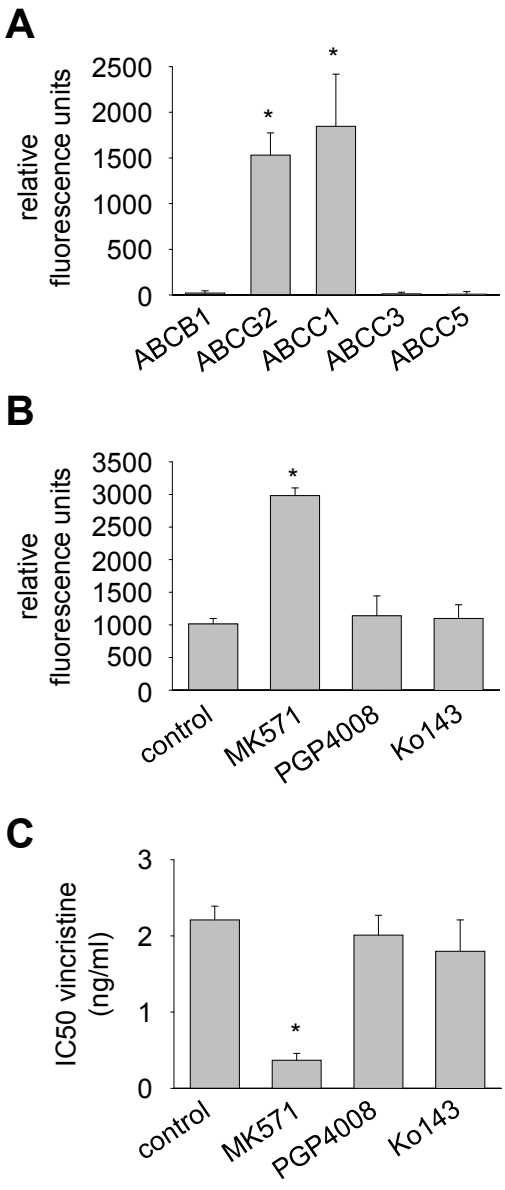
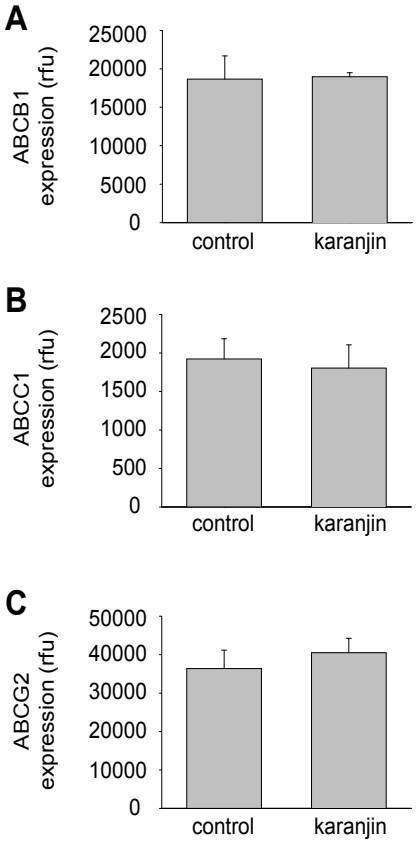


Suppl. Figure 1. G62 as ABCC1 model. A) expression of different ABC transporters on G62 cells determined by flow cytometry. Values are presented after subtraction of respective isotype controls. * $P < 0.05$ relative to isotype control B) fluorescence of the ABCC1 substrate 5-CFDA 10µM in non-treated G62 cells (control) or cells treated with MK571 10µM (ABCC1 inhibitor), PGP4008 2.5 µM (ABCB1 inhibitor), or fumitremorgin C 10µM (ABCG2 inhibitor). * $P < 0.05$ relative to control C) vincristine concentrations that reduce G62 cell viability by 50% (IC50). G62 cells were treated with vincristine alone (control) or with vincristine in the presence of MK571 10µM, PGP4008 2.5 µM, or Ko143 10µM. * $P < 0.05$ relative to control.



Suppl. Figure 2. ABC transporter expression in the presence of karanjin. A) ABCB1 expression in non-treated UKF-NB-3^{ABCB1} cells (control) or in UKF-NB-3^{ABCB1} cells treated with karanjin 40µM for 5 days. B) ABCC1 expression in non-treated G62 cells (control) or in G62 cells treated with karanjin 40µM for 5 days. C) ABCG2 expression in non-treated UKF-NB-3^{ABCG2} cells or in UKF-NB-3^{ABCG2} cells treated with karanjin 40µM for 5 days.

Suppl. Figure 3. ABCB1 docking studies of karanjin, apigenin, genistein, and naringenin.

INTRODUCTION

The docking study of karanjin, apigenin, genistein, and naringenin with ABCB1 was carried out using 3D structures of mouse Abcb1a. The x-ray structures of the mouse Abcb1a was downloaded from the protein data bank (PDB codes 3G60, 3G61 and 5GU) [http://www.rcsb.org]. It should be noted that two of the Abcb1a structures, 3G60, 3G61, are co-crystallised with stereo-isomers of cyclic hexapeptide inhibitors, cyclic-tris-(R)-valineselenazole (QZ59-RRR) and cyclic-tris-(S)-valineselenazole (QZ59-SSS). Docking was carried out using MOE software (MOE, version 2011.10, Chemical Computing Group Inc. Montreal, Canada) as below.

A Abcb1a structure was loaded into the software, where the crystal parameters were retained and all

atoms of the protein (Abcb1a) were protonated and titrated using default parameters of the software. To prepare the flavonoids for docking, atomic charge and energy minimisation calculation was performed using SCF optimisation (AM1 Hamiltonian). In MOE dock panel, the placement method was Triangle Matcher, scoring methodology was set to London dG as the first and the second scoring functions, refinement methodology was set to MMFF94 force field and, finally, 30 best scoring poses with the mean energies and backbone root mean square deviation (RMSD) were retained. The binding site was defined in MOE software as 1) QZ59-RRR binding site in 3G60 protein, 2) verapamil binding site for 3G5U, 3) upper ligand binding site for QZ59-SSS in 3G61 and, 4) lower ligand binding site for QZ59-SSS in 3G61. Default parameters were used for ligand interactions diagram with energy cut-off for hydrogen bonding and ionic interactions of -0.5 kcal/mol. Maximum distance for non-bonded atoms was 4.5 angstrom.

RESULTS

Docking energies of the top scoring poses for interaction with four Abcb1a structure/binding site has been presented in Table 1.

Table 1 shows that interactions are better with the binding site of QZ59-RRR in 3G60 structure. Table 2 shows the scores of the second and third best docking poses with this binding site and the RMSD values of the top poses. The lowest calculated docking energies are presented in Table 2.

Table 2. Protein interaction energies (kcal/mol) of four top poses of docking Abcb1a with flavonoids and the lowest RMSD values between the first and second poses for karanjin and naringenin, and between the third and fourth poses for apigenin and genistein.

Table 3 shows the most important residues involved in the flavonoid-Abcb1a interaction according to the 2D-ligand interaction diagram of the top pose in ligand-enzyme docking. It must be noted that Phe 71, Met 68, Phe 974 are less important in the interaction compared to other residues in in the table.

DISCUSSION

All the flavonoids here apart from karanjin contain two hydroxyl groups in the A ring at positions 5 and 7, and in addition there is a hydroxyl group present in ring C. Hydroxyl groups can decrease the lipophilicity while it is a known fact that ABCB1 preference is for lipophilic compounds (Omote et al, 2006; Gatlik-Landwojtowicz et al, 2006; Gottesman et al, 1993). The yellow colour on the surface of the binding cavity in Abcb1a in Figure 1 indicates hydrophobic properties of the surface and it could be appropriate match with the hydrophobic rings of the flavonoids.

All four flavonoids tested in this study have shown good affinities for the active site of receptor (Table 1). Docking energies of Table 1 shows that apigenin has the highest affinity to Abcb1a among the tested flavonoids. Previous *in vivo* studies by Conseil et al. also show that flavones like apigenin bind more strongly to ABCB1 than flavanones such as naringenin or isoflavones such as genistien. On the other hand, they show that highly hydrophilic flavonoid, rutin, has a very low affinity interaction with ABCB1 (Conseil et al, 1998).

The residues Phe 724, Phe 332, Val 978, Phe 728, Tyr 303 in all four flavonoids are appointed as the key residues contributing to the affinity energy of the complexes studied. All complexes have shown relatively strong bonds with ring A. Furthermore, for apigenin and naringenin the interaction included strong hydrogen bonds and this could be the reason for higher docking energy. Broccatelli and his colleges previously report importance of hydrogen bonding in the interaction of flavonoid with ABCB1 (Broccatelli et al. 2011).

Three of the residues which bind to flavonoids in this study, are also the main residues in co-crystallized structure and docked models of P-gp for binding to QZ59-RRR.These are Phe 728, Phe 974 and Val 978.

In this report we have shown that docking method is able to predict the binding of a small group of flavonoids and have provided good understanding of the interaction between the receptor (ABCB1) and inhibitors.

Table 1. Top pose docking scores for flavonoids binding to Abcb1a.

Flavonoids	Abcb1a structure used for docking			
	3G60 (RRR)	3G5U(using verapamil binding site)	3G61 using Upper QZ59-SSS (Smaller)	3G61 using Upper QZ59-SSS (Bigger)
Karanjin	-10.36	-7.15	-8.92	-7.58
Apigenin	-12.98	-10.20	-10.55	-9.98
Genistein	-10.88	-9.30	-10.54	-8.63
Naringenin	-12.43	-9.47	-10.48	-8.96

Table 2.

Flavonoids	Docking energy of top four scoring poses (left to right)				RMSD (Å)
Karanjin	-10.36	-10.19	-10.16	-10.06	2.15
Apigenin	-12.98	-11.83	-11.76	-11.13	2.68
Genistein	-10.88	-10.38	-10.37	-10.28	2.90
Naringenin	-12.43	-12.18	-11.87	-11.63	2.16

Table 3. Most important residues for docking of karanjin, apigenin, genistein and naringenin.

Flavonoids	Interacting and implying residues in first and second poses
Karanjin	Phe 724, Phe 332, Val 978, Phe 728, Tyr 303, Phe 71, Met 68, Phe 974
Apigenin	Phe 724, Phe 332, Val 978, Phe 728, Tyr 303, Phe 71, Phe 974
Genistein	Phe 724, Phe 332, Val 978, Phe 728, Tyr 303, Phe 71, Phe 974
Naringenin	Phe 724, Phe 332, Val 978, Phe 728, Tyr 303, Phe 71, Phe 974

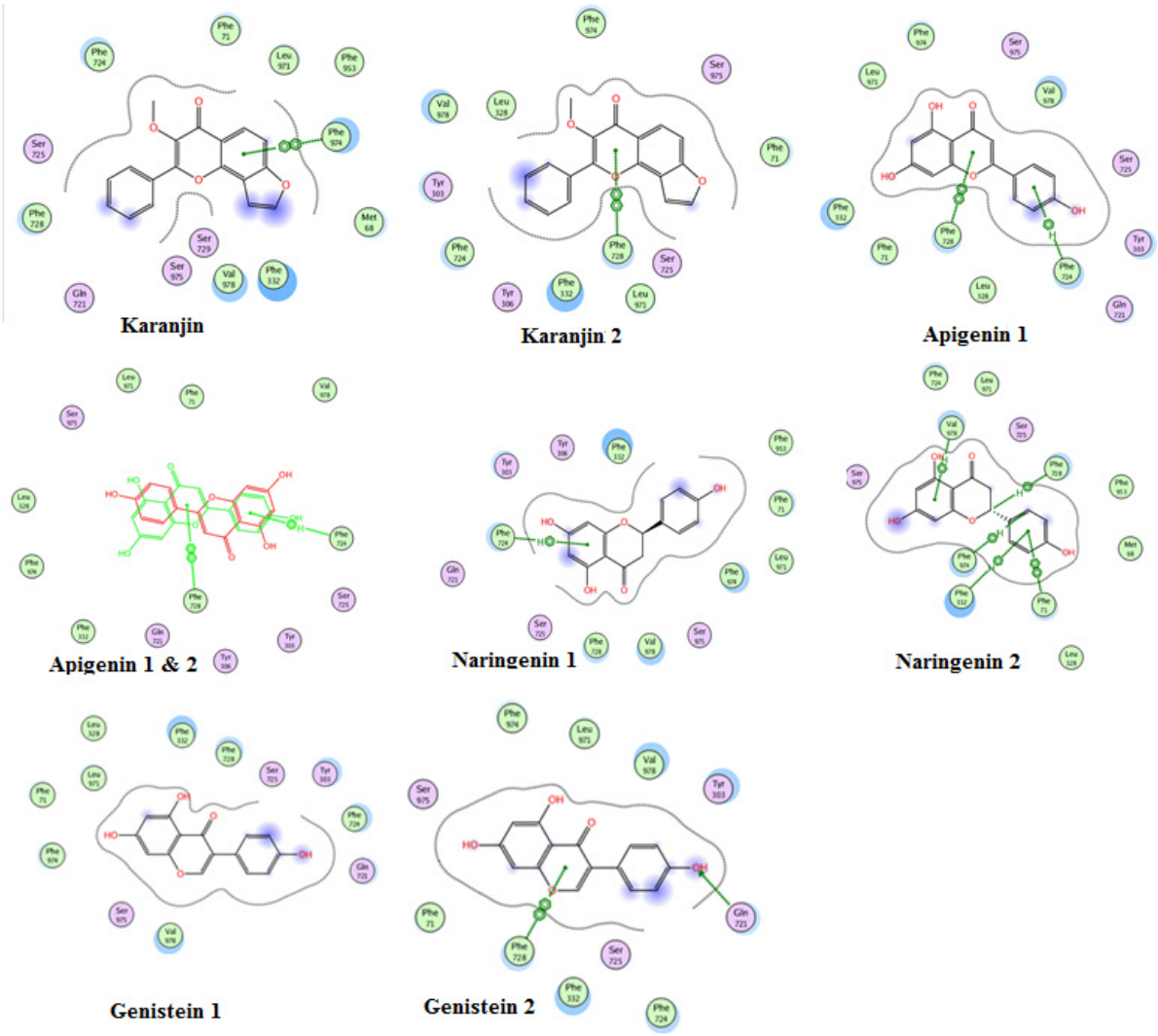


Figure 1. 2D Ligand interaction diagrams for the top and the second top pose for docking karanjin, apigenin, naringenin and genistein into the binding site of the mouse Abcb1a (3G60) using MOE software; the polar and non-polar amino-acids are shown in pink and green circles; hydrogen bonding is indicated by green dotted arrows; proximity contour are dotted lines surrounding the ligand and indicate the shape of the binding site and available space to the more outward-facing parts of the ligand; solvent exposure zone for the ligands have been indicated by violaceous areola (for example in benzofuran and chromene in karanjin).

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Suppl. Table 1

UKF-NB-3ABCB1				
			in the presence of karanjin	
karanjin	Cell viability	IC50 vincristine	IC50 vincristine	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
10	77.57 ± 10.11	7.61 ± 0.83	1.33 ± 0.19*	5.72
			in the presence of apigenin	
apigenin	Cell viability	IC50 vincristine	IC50 vincristine	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
10	80.50 ± 10.11	7.61 ± 0.83	3.57 ± 0.65*	2.13
			in the presence of genistein	
genistein	Cell viability	IC50 vincristine	IC50 vincristine	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
10	59.77 ± 11.93	7.61 ± 0.83	4.67 ± 0.91*	1.63
			in the presence of naringenin	
apigenin	Cell viability	IC50 vincristine	IC50 vincristine	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
10	65.75 ± 8.29	7.61 ± 0.83	4.48 ± 0.80*	1.70
			in the presence of PGP4008	
PGP4008**	Cell viability	IC50 vincristine	IC50 vincristine	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
2.5	80.39 ± 10.74	7.61 ± 0.83	0.10 ± 0.02*	76.10
* P < 0.05 relative to vincristine alone				
** Specific P-glycoprotein inhibitor serving as control				

UKF-NB-3ABCB1				
			in the presence of karanjin	
karanjin	Cell viability	IC50 vincristine	IC50 vincristine	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
0.625	100.01 ± 10.83	7.61 ± 0.83	6.90 ± 1.12	1.10
1.25	87.47 ± 12.25	7.61 ± 0.83	3.03 ± 0.40*	2.51
2.5	82.52 ± 17.14	7.61 ± 0.83	2.16 ± 0.36*	3.52
5	84.88 ± 9.19	7.61 ± 0.83	1.94 ± 0.26*	3.92
10	77.57 ± 10.11	7.61 ± 0.83	1.33 ± 0.19*	5.72
20	76.13 ± 9.52	7.61 ± 0.83	0.09 ± 0.03*	84.56
40	62.24 ± 6.01	7.61 ± 0.83	0.05 ± 0.01*	152.20
			in the presence of PGP4008	
PGP4008**	Cell viability	IC50 vincristine	IC50 vincristine	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
2.5	80.39 ± 10.74	7.61 ± 0.83	0.10 ± 0.02*	76.10
* P < 0.05 relative to vincristine alone				
** Specific ABCB1 inhibitor serving as control				

G62				
			in the presence of karanjin	
karanjin	Cell viability	IC50 vincristine	IC50 vincristine	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
10	93.81 ± 6.17	2.72 ± 0.35	0.57 ± 0.12*	4.77
			in the presence of apigenin	
apigenin	Cell viability	IC50 vincristine	IC50 vincristine	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
10	87.51 ± 19.16	2.72 ± 0.35	1.24 ± 0.43*	2.19
			in the presence of genistein	
genistein	Cell viability	IC50 vincristine	IC50 vincristine	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
10	85.17 ± 12.42	2.72 ± 0.35	3.35 ± 0.62	0.81
			in the presence of naringenin	
naringenin	Cell viability	IC50 vincristine	IC50 vincristine	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
10	91.05 ± 8.29	2.72 ± 0.35	2.69 ± 0.74	1.01
			in the presence of MK571	
MK571**	Cell viability	IC50 vincristine	IC50 vincristine	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
10	99.47 ± 2.84	2.72 ± 0.35	0.76 ± 0.11*	3.58
* P < 0.05 relative to vincristine alone				
** Specific MRP1 inhibitor serving as control				

UKF-NB-3				
			in the presence of karanjin	
karanjin	Cell viability	IC50 vincristine	IC50 vincristine	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
0.625	99.17 ± 4.26	0.14 ± 0.03	0.14 ± 0.04	1.00
1.25	96.00 ± 12.16	0.14 ± 0.03	0.13 ± 0.04	1.08
2.5	98.69 ± 12.09	0.14 ± 0.03	0.11 ± 0.03	1.27
5	95.22 ± 9.21	0.14 ± 0.03	0.11 ± 0.04	1.27
10	70.63 ± 10.82	0.14 ± 0.03	0.12 ± 0.03	1.17
20	64.01 ± 8.83	0.14 ± 0.03	0.10 ± 0.03	1.40
40	60.13 ± 9.45	0.14 ± 0.03	0.10 ± 0.05	1.40
			in the presence of PGP4008	
PGP4008**	Cell viability	IC50 vincristine	IC50 vincristine	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
2.5	72.55 ± 15.90	0.14 ± 0.03	0.09 ± 0.05	1.56
* P < 0.05 relative to vincristine alone				
** Specific ABCB1 inhibitor serving as control				

UKF-NB-3ABCG2				
			in the presence of karanjin	
karanjin	Cell viability	IC50 mitoxantrone	IC50 mitoxantrone	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
10	88.03 ± 16.05	49.47 ± 10.60	1.50 ± 0.21*	32.98
			in the presence of apigenin	
apigenin	Cell viability	IC50 mitoxantrone	IC50 mitoxantrone	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
10	83.39 ± 14.36	49.47 ± 10.60	2.89 ± 0.83*	17.11
			in the presence of genistein	
genistein	Cell viability	IC50 mitoxantrone	IC50 mitoxantrone	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
10	71.11 ± 16.84	49.47 ± 10.60	2.14 ± 0.53*	23.11
			in the presence of naringenin	
naringenin	Cell viability	IC50 mitoxantrone	IC50 mitoxantrone	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
10	88.17 ± 15.54	49.47 ± 10.60	7.54 ± 1.21*	6.56
			in the presence of Ko143	
Ko143**	Cell viability	IC50 mitoxantrone	IC50 mitoxantrone	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
10	89.01 ± 12.14	49.47 ± 10.60	0.34 ± 0.06*	145.50
* P < 0.05 relative to mitoxantrone alone				
** Specific BCRP1 inhibitor serving as control				

UKF-NB-3Cer2				
			in the presence of karanjin	
karanjin	Cell viability	IC50 vincristine	IC50 vincristine	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
0.625	86.33 ± 14.21	0.46 ± 0.19	0.63 ± 0.07	0.73
1.25	82.69 ± 20.09	0.46 ± 0.19	0.47 ± 0.04	0.98
2.5	79.93 ± 22.98	0.46 ± 0.19	0.38 ± 0.10	1.21
5	76.74 ± 12.80	0.46 ± 0.19	0.37 ± 0.11	1.24
10	75.02 ± 11.85	0.46 ± 0.19	0.35 ± 0.15	1.31
20	73.22 ± 9.19	0.46 ± 0.19	0.35 ± 0.12	1.31
40	71.39 ± 17.19	0.46 ± 0.19	0.33 ± 0.10	1.39
			in the presence of PGP4008	
PGP4008**	Cell viability	IC50 vincristine	IC50 vincristine	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
2.5	74.62 ± 11.13	0.46 ± 0.19	0.40 ± 0.08	1.15
* P < 0.05 relative to vincristine alone				
** Specific ABCB1 inhibitor serving as control				

Suppl. Table 2

Cont'd …

Suppl. Table 2, Cont'd

UKF-NB-3rVCR10				
			in the presence of karanjin	
karanjin	Cell viability	IC50 vincristine	IC50 vincristine	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
0.625	105.63 ± 9.17	56.82 ± 7.04	48.12 ± 9.65	1.18
1.25	94.58 ± 6.72	56.82 ± 7.04	22.47 ± 2.33*	2.53
2.5	102.93 ± 11.96	56.82 ± 7.04	18.23 ± 0.96*	3.11
5	97.22 ± 8.54	56.82 ± 7.04	10.71 ± 1.09*	5.31
10	90.49 ± 7.69	56.82 ± 7.04	5.89 ± 0.80*	9.65
20	89.61 ± 11.43	56.82 ± 7.04	0.70 ± 0.16*	81.17
40	68.98 ± 10.84	56.82 ± 7.04	0.52 ± 0.03*	109.27
			in the presence of PGP4008	
PGP4008**	Cell viability	IC50 vincristine	IC50 vincristine	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
2.5	86.50 ± 12.64	56.82 ± 7.04	1.01 ± 0.16*	56.26
* P < 0.05 relative to vincristine alone				
** Specific ABCB1 inhibitor serving as control				

G62				
			in the presence of karanjin	
karanjin	Cell viability	IC50 vincristine	IC50 vincristine	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
0.625	97.42 ± 2.00	2.72 ± 0.35	2.67 ± 0.21	1.02
1.25	96.71 ± 5.57	2.72 ± 0.35	2.83 ± 0.27	0.96
2.5	93.69 ± 4.00	2.72 ± 0.35	2.51 ± 0.52	1.08
5	95.16 ± 5.02	2.72 ± 0.35	1.16 ± 0.10*	2.34
10	93.81 ± 6.17	2.72 ± 0.35	0.57 ± 0.12*	4.77
20	92.73 ± 3.94	2.72 ± 0.35	0.37 ± 0.04*	7.35
40	66.82 ± 5.62	2.72 ± 0.35	0.36 ± 0.16*	7.56
			in the presence of MK571	
MK571**	Cell viability	IC50 vincristine	IC50 vincristine	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
10	99.47 ± 2.84	2.72 ± 0.35	0.76 ± 0.11*	3.58
* P < 0.05 relative to vincristine alone				
** Specific ABCC1 inhibitor serving as control				

PC3rVCR20				
			in the presence of karanjin	
karanjin	Cell viability	IC50 vincristine	IC50 vincristine	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
0.625	101.12 ± 2.72	31.06 ± 4.87	30.68 ± 1.81	1.01
1.25	97.42 ± 3.59	31.06 ± 4.87	29.37 ± 1.30	1.06
2.5	104.00 ± 10.00	31.06 ± 4.87	25.89 ± 2.62	1.20
5	102.50 ± 12.50	31.06 ± 4.87	17.02 ± 1.57*	1.82
10	100.30 ± 2.71	31.06 ± 4.87	11.75 ± 0.91*	2.64
20	98.98 ± 4.03	31.06 ± 4.87	5.61 ± 1.83*	5.54
40	53.21 ± 5.98	31.06 ± 4.87	2.46 ± 0.60*	12.63
			in the presence of MK571	
MK571**	Cell viability	IC50 vincristine	IC50 vincristine	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
10	115.00 ± 7.00	31.06 ± 4.87	8.01 ± 2.03*	3.88
* P < 0.05 relative to vincristine alone				
** Specific ABCC1 inhibitor serving as control				

UKF-NB-3ABCG2				
			in the presence of karanjin	
karanjin	Cell viability	IC50 mitoxantrone	IC50 mitoxantrone	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
0.15625	103.45 ± 11.92	49.47 ± 10.60	39.91 ± 6.76	1.24
0.3125	100.62 ± 12.00	49.47 ± 10.60	23.22 ± 4.69*	2.13
0.625	98.13 ± 10.01	49.47 ± 10.60	10.74 ± 1.21*	4.61
1.25	91.95 ± 7.88	49.47 ± 10.60	5.49 ± 0.72*	9.01
2.5	78.97 ± 14.62	49.47 ± 10.60	4.56 ± 0.99*	10.85
5	86.32 ± 8.91	49.47 ± 10.60	3.53 ± 0.66*	14.01
10	88.03 ± 16.05	49.47 ± 10.60	1.50 ± 0.21*	32.98
20	83.73 ± 15.22	49.47 ± 10.60	0.23 ± 0.02*	216.98
40	64.31 ± 11.18	49.47 ± 10.60	0.07 ± 0.03*	706.71
			in the presence of Ko143	
Ko143**	Cell viability	IC50 mitoxantrone	IC50 mitoxantrone	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
10	89.01 ± 12.14	49.47 ± 10.60	0.34 ± 0.06*	145.50
* P < 0.05 relative to mitoxantrone alone				
** Specific ABCG2 inhibitor serving as control				

UKF-NB-3				
			in the presence of karanjin	
karanjin	Cell viability	IC50 mitoxantrone	IC50 mitoxantrone	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
0.15625	103.33 ± 8.37	0.18 ± 0.04	0.15 ± 0.02	1.20
0.3125	105.72 ± 9.00	0.18 ± 0.04	0.20 ± 0.06	0.90
0.625	99.16 ± 9.75	0.18 ± 0.04	0.20 ± 0.03	0.90
1.25	100.62 ± 12.93	0.18 ± 0.04	0.15 ± 0.05	1.20
2.5	98.91 ± 11.39	0.18 ± 0.04	0.16 ± 0.02	1.13
5	86.12 ± 15.19	0.18 ± 0.04	0.17 ± 0.03	1.06
10	77.72 ± 9.94	0.18 ± 0.04	0.15 ± 0.04	1.20
20	75.76 ± 10.44	0.18 ± 0.04	0.15 ± 0.02	1.20
40	70.35 ± 8.25	0.18 ± 0.04	0.16 ± 0.03	1.13
			in the presence of Ko143	
Ko143*	Cell viability	IC50 mitoxantrone	IC50 mitoxantrone	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
10	87.59 ± 7.99	0.18 ± 0.04	0.15 ± 0.04	1.20
* Specific ABCG2 inhibitor serving as control				

UKF-NB-3iG2				
			in the presence of karanjin	
karanjin	Cell viability	IC50 mitoxantrone	IC50 mitoxantrone	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
0.15625	106.88 ± 8.39	0.19 ± 0.04	0.19 ± 0.03	1.00
0.3125	98.98 ± 11.00	0.19 ± 0.04	0.21 ± 0.04	0.90
0.625	94.57 ± 8.25	0.19 ± 0.04	0.19 ± 0.03	1.00
1.25	101.01 ± 10.82	0.19 ± 0.04	0.14 ± 0.04	1.36
2.5	104.84 ± 7.73	0.19 ± 0.04	0.17 ± 0.02	1.12
5	95.61 ± 12.21	0.19 ± 0.04	0.20 ± 0.03	0.95
10	91.38 ± 10.71	0.19 ± 0.04	0.19 ± 0.02	1.00
20	85.11 ± 9.98	0.19 ± 0.04	0.17 ± 0.05	1.12
40	82.04 ± 8.20	0.19 ± 0.04	0.17 ± 0.04	1.12
			in the presence of Ko143	
Ko143*	Cell viability	IC50 mitoxantrone	IC50 mitoxantrone	fold sensitisation
(μM)	(% control)	(ng/ml)	(ng/ml)	
10	80.32 ± 15.09	0.19 ± 0.04	0.16 ± 0.04	1.19
* Specific ABCG2 inhibitor serving as control				

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