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Application of Fluorescent Dye Substrates for Functional Characterization of ABC Multidrug Transporters at a Single Cell Level

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Abstract

ABC multidrug transporters are key players in cancer multidrug resistance and in determining the ADME-Tox properties of drugs and xenobiotics. The most sensitive and specific detection of these transporters is based on functional assays. Assessment of the transporter-dependent reduction of cellular uptake of the fluorescent dyes, such as Hoechst 33342 (Ho) and more recently DyeCycle Violet (DCV), have been widely advocated for the characterization of both ABCB1 and ABCG2 multidrug transporters. Detailed comparison of these supravital DNA-binding dyes revealed that DCV is less toxic to ABCG2- and ABCB1-expressing cells than Ho. ATPase measurements imply that DCV and Ho are similarly handled by ABCB1, whereas ABCG2 seems to transport DVC more effectively. In addition, we have developed an image-based high content microscopy screening method for simultaneous in situ measurement of the cellular activity and expression of the ABCG2 multidrug transporter. We demonstrated the applicability of this method for identifying ABCG2-positive cells in heterogeneous cell population by a single dye uptake measurement. These results may promote multidrug transporter studies at a single cell level and allow the quantitative detection of clinically important drug-resistant sub-populations. © 2016 International Society for Advancement of Cytometry

Key terms

functional transporter assay; multidrug transporters; ABCG2; ABCB1; DyeCycle Violet; Hoechts 33342; high content screening and analysis

MULTIDRUG resistance is a major problem in chemotherapy of cancer or in prolonged viral infections. An important cause of the emergence of cellular multidrug resistance is the increased expression of ABC multidrug transporters (1,2). Three human ABC proteins, ABCB1, ABCG2, and ABCC1 are the key membrane proteins responsible for an increased outward drug transport in most multidrug-resistant cells. These transporters are members of the ancient ABC transporter protein family, performing primary, ATP-dependent active transport processes. The human multidrug transporters consist of conserved cytoplasmic ABC domains, responsible for the ATP binding and hydrolysis, and multipass membrane region(s) involved in drug substrate binding and outward drug transport (3,4). The above mentioned three human multidrug transporters show promiscuous drug binding characteristics and have partially overlapping substrate specificities. Prediction of interactions between ABC multidrug transporters and drugs is a challenging task, because the structure of these proteins is not known at an atomic level, and the drug-protein interaction site is probably a large and versatile interface (5).

Both in vitro and in vivo studies clearly indicate that in multidrug-resistant cells the therapeutically effective drug concentrations strongly depend on the actual, func-

tional expression levels of the ABC multidrug transporters. In humans, ABC multidrug transporters are expressed in all major tissue barriers; and ABCG2 is specifically expressed in various stem cells. Moreover, in normal or cancer cell populations the expression levels of drug transporters highly vary from cell to cell. An important example for this variability is the suggested presence of cancer stem cells in malignant tumors (1,6–8). It has been widely documented that cancer stem cells express relatively high, protective levels of multidrug transporters, especially the ABCG2 and/or the ABCB1 proteins, providing the so-called side-population (SP) phenotype (2). Thus, a single cell functional detection of the ABC multidrug transporter proteins, preferably in live and surviving cells, has a major importance.

In the present report we provide a detailed comparison of the cellular interactions of two fluorescent supravital dyes, transported substrates of both ABCB1 and ABCG2. The Hoechst 33342 (Ho) dye has already been widely applied in cellular studies, while the recently developed DyeCycle Violet (DCV) has been less characterized in this respect (9–13). Both DCV and Ho are DNA binding dyes, and become fluorescent only in a DNA-bound form. This property promotes the use of these dyes in continuous dye uptake measurements, or even in in situ microscopy settings, without washing of the cells. DCV was originally developed as a cell cycle marker by Molecular Probes™, and the fluorescence properties render it more applicable than Ho for measurements by using violet (exc. 405 nm) lasers, available in most cell biology instruments. Although DCV has already been used to identify and sort drug-resistant side population cells based on a transporter related reduction of DCV uptake (9,10,13), a detailed analysis of DCV-transporter interactions has not been provided.

In the present study, we performed detailed direct ABC transporter interaction assays by using both dyes, examined their cellular toxicity in parental and drug-resistant cell populations, and followed the modulation of DCV and Ho accumulation by specific transporter inhibitors. Moreover, we have performed high content screening assays to reveal the in situ kinetics of the modulation of dye uptake by ABCG2, parallel with a specific antibody staining of the transporter. These approaches allow the identification of various functional ABCG2 expression levels in a mixed, live cell population.

Materials and Methods

Materials

Unless otherwise stated, all reagents were obtained from Sigma–Aldrich. DyeCycle Violet, FIX & PERM® Cell Fixation & Cell Permeabilization Kit and Hoechst 33342 were obtained from Thermo Fisher Scientific. The 5D3 antibody was purified from 5D3 hybridoma cell line (a kind gift of Dr. Brian Sorrentino) and labeled with AlexaFluor647 as described previously (14). The MRK-16 antibody was obtained from Kamiya Biomedical Company. Ko143 was obtained from Tocris Bioscience.

Cell Lines

Previously we have generated ABCB1- and ABCG2-overexpressing K562, and ABCB1-, ABCC1-, and ABCG2-overexpressing A431 cell lines by retroviral transduction (15,16). The expression and function of ABC transporters in these cell lines were examined by antibody staining and dye extrusion assays, respectively, as detailed below. Parental, ABCB1- and ABCG2-expressing A431 cells were also modified to express cytosolic mOrange fluorescent protein (ex: 548, em: 562) by lentiviral transduction. Generation of cell lines was performed with the support of Creative Cell (Budapest, Hungary). Sf9 insect cells used for ATPase assays were infected with recombinant baculoviruses harboring ABCB1 and ABCG2 as described previously (17).

Membrane ATPase Measurements

ATPase activity of ABC transporters was measured in membrane vesicles prepared from insect (Sf9) cells expressing human wild type ABCB1 or ABCG2 (17). In order to ensure maximal ABCG2 activity, Sf9 membranes were loaded with cholesterol to the level of the mammalian type membranes, as described previously (18). Vanadate-sensitive ATPase activity of ABCB1 and ABCG2 was measured by a colorimetric reaction (19).

Cytotoxicity Assay

DCV and Ho cytotoxicity was measured in viability assays by using K562 and A431 cells expressing the ABCB1 or ABCG2 proteins. Viability assays were carried out in 96-well plates. 1×10^4 K562 cells/well were seeded in RPMI media, the DCV or the Ho dye was added in increasing concentrations, and the plates were incubated for 72 h at 37°C. For measuring cell viability, the PrestoBlue reagent (Life Technologies) was used as described earlier (20). In the case of A431 cells, 4×10^3 cells were seeded onto 96-well plates 24 h prior to the addition of the examined dyes. Following a 72 h incubation period, the viability was determined by the mOrange fluorescence of the cells. Preliminary experiments demonstrated close correlation between the amount of cells and the measured mOrange fluorescence (data not shown). Fluorescence (579/25 nm) was detected by a Perkin Elmer Victor X3 2030 Multilabel Plate Reader at 535/25 nm excitation, both for PrestoBlue and mOrange. Fluorescence of the untreated samples considered as 100%. Cytotoxicity was determined by the IC_{50} values, calculated by Origin software (OriginLab) from the viability curves.

Flow Cytometry for Fluorescent Dye Uptake Measurements and for Immunostaining

Cell surface expression of the ABC transporters was followed by labeling the cells with monoclonal antibodies that recognize an external epitope of the given transporter. Anti-ABCB1 MRK-16 and anti-ABCG2 5D3 primary antibodies were used for this detection. 5×10^5 K562 or A431 cells were incubated with the primary antibody (1 μg/ml) for 30 min at 37°C. Labeling ABCG2 was performed in the presence of 5 μM Ko143 inhibitor to obtain maximal signal with the 5D3 conformation-sensitive antibody. After washing, the cells were

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further incubated with a secondary, Brilliant Violet 421 conjugated anti-mouse IgG antibody (BioLegend, 2 μg/ml). Fluorescence was detected by an Attune Acoustic Focusing Cytometer (B/V, Applied Biosystems). Functional activity of the multidrug transporter proteins was measured by dye uptake assays in flow cytometry. 5×10^5 cells were preincubated in the absence or presence of the specific inhibitor of the transporter protein in HPMI medium (120 mM NaCl, 5 mM KCl, 400 μM $MgCl_2$, 40 μM $CaCl_2$, 10 mM Hepes, 10 mM $NaHCO_3$, 10 mM glucose, and 5 mM Na_2HPO_4) for 10 min at 37°C. After preincubation, the cells were subjected to DCV (1 μM) or Ho (1 μM) for 20 min at 37°C. The uptake was terminated by the addition of ice-cold PBS. After centrifugation the cells were resuspended in PBS and fluorescence of the samples was measured at 450/40 nm in an Attune flow cytometer using 405 nm laser for excitation. The transport activity of ABC transporters was characterized by the "transport activity factor" calculated as follows: $(F_{100} - F_0)/F_{100} \times 100$, where F_0 is the fluorescence (median values) of DCV/Ho-treated cells in the absence of an inhibitor; F_{100} is the fluorescence (median values) in the presence of the inhibitor (21). As transporter inhibitors, elacridar (5 μM), Ko143 (5 μM), and MK-571 (100 μM) were used for ABCB1, ABCG2, and ABCC1, respectively.

High Content Screening Measurement of DyeCycle Violet Uptake

A431 and A431/ABCG2 cells were used for High Content Screening (HCS) measurements. To generate mixed populations, parental and ABCG2-expressing A431 cells were mixed in a ratio of 98:2. The cells were seeded into a 96-well plate (Cellstar, Greiner) at 4×10^3 cells/well density in 100 µl α-MEM cell culture medium 16 h prior to the experiment. Dye uptake measurement was carried out in an ImageXpress Micro XLS High Content System (Molecular Devices, Sunnyvale, CA, USA) with a Nikon CFI Super Plan Fluor ELWD ADM 20× objective. The cells were incubated with 1 µM DCV for 90 min at 37°C, and then subjected to 5 µM Ko143 for an additional 60 min. The fluorescence of DCV (447/60 nm) were acquired every 5 min at 377/50 nm excitation.

Immunodetection of ABCG2 by High Content Screening

Following the DCV uptake measurement, the cells were immunolabeled for ABCG2 as follows. Cells were washed three times with Dulbecco's modified phosphate-buffered saline (DPBS) and fixed for 15 min at room temperature with Fix&Perm A solution (Thermo F.S.). After four washes with DPBS, non-specific antibody binding was blocked by Fix&Perm B solution for 1 h, and the samples were stained by AlexaFluor647-conjugated 5D3 anti-ABCG2 antibody (2 µg/ml final concentration) for 1 h. After repeated washes with DPBS, the plate was imaged by the High Content Screening System. The fluorescence signals for DCV (447/60 nm) and AlexaFluor647-labelled antibody staining (692/40 nm) were acquired at 377/50 and 628/40 nm excitations, respectively.

High Content Data Analysis

To identify even a very small fraction of ABCG2-expressing cells in a heterogeneous cell population, the ImageXpress Micro XLS Widefield High Content Analysis System and the MetaXpress High-Content Image Acquisition and Analysis Software were used. Each well, containing a few hundreds of cells, was imaged every 5 min throughout the experiment as described above. During the analysis, an identification number (Object ID) was assigned to each cell. The cells were tracked with Multi Dimensional Motion Analysis (MDMA) application, which can follow the movement of the cells in time and provide the average fluorescence intensity of each nucleus at every time point using a series of mask images. To generate a measure of DCV uptake for each cell, the fluorescence intensities of the nuclei (F_1 and F_2) were determined at two selected time points, 15 and 60 min, respectively, after the addition of Ko143 (carried out after a 90 min incubation with DCV). The "DCV transport ratio" for each cell with the given Object ID was calculated as F_2/F_1. Using the last mask image created by the MDMA application on the basis of the blue fluorescence, the cells positive for 5D3 antibody staining was identified by the Multi Wavelength Cell Scoring (MWCS) application. MDMA data for DCV uptake and MWCS data for immunostaining were compared to investigate the correlation of these independent detections.

Statistical significance was performed using paired Student's t-test. $P < 0.01$ was considered as significant difference.

Results

DCV and Ho Directly Interact With Both ABCB1 and ABCG2 According to the ATPase Activity Assay

ABC multidrug transporters use the energy of ATP binding and hydrolysis for drug extrusion; ATPase activity and drug transport are coupled processes. Therefore, drug substrates and inhibitors significantly modify the ATPase activity of the transporters in a concentration-dependent manner, allowing an estimation of direct interactions with drugs (19). In isolated membrane preparations containing the transporter proteins, both ABCB1 and ABCG2 have a basal ATPase activity (without added substrate drug), which can be either stimulated or inhibited by the interacting drugs. Rapidly transported substrates usually activate ABC-ATPase, while inhibitors or slowly transported compounds reduce the basal and/or the drug-stimulated ATPase activity (17–19).

In this study the ATPase activity was measured in human ABCG2- or ABCB1-containing cell membrane vesicles, prepared from transporter-overexpressing Sf9 insect cells. The relatively low basal ATPase activity of ABCB1 was not significantly influenced by either DCV or Ho (Fig. 1A). In contrast, drug-stimulated ABCB1-ATPase activity in the presence of well-known substrates, including reversin 121 (or verapamil, data not shown) was inhibited by both dyes in a concentration-dependent manner (Fig. 1A). We found no significant differences between DCV and Ho in terms of their concentration-dependent reduction of this ATPase activity, with approximate IC_{50} values of 3–5 μM (Fig. 1A). F1

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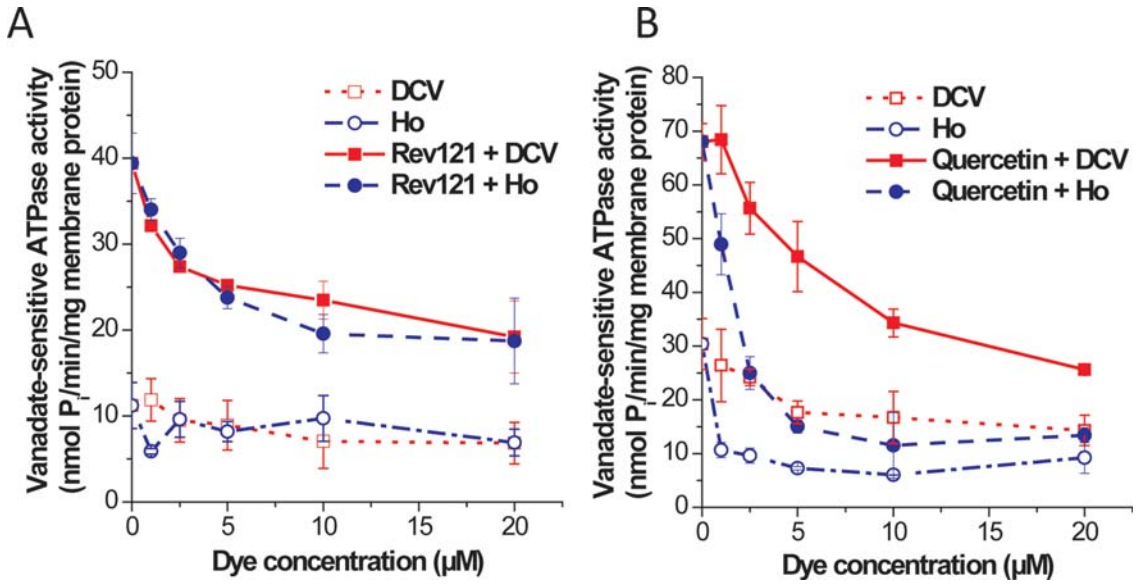

Figure 1. Influence of DCV and Ho on the ATPase activity of ABCB1 and ABCG2 transporters. The modulatory effects of DCV and Ho on the basal and stimulated ATPase activities of ABCB1 (**A**) and ABCG2 (**B**) were studied in isolated Sf9 membrane preparations containing the investigated transporter. In addition, cholesterol-loaded Sf9 membrane vesicles were used for the ABCG2 assays. For the stimulated ATPase activity of ABCB1 and ABCG2, the experiments were performed in the presence of 1 μM Reversin 121 and 1 μM Quercetin, respectively. The data points show the average of three independent experiments, the error bars represent SD values.

In the case of the ABCG2 protein, having a higher basal ATPase activity, both the basal activity and the drug-stimulated ATPase (induced by quercetin) were inhibited by both DCV and Ho (Fig. 1B). The approximate IC_{50} value for DCV (5 μM) was higher than that for Ho (about 1.5 μM), suggesting a stronger interaction, which predicts slower transport of Ho by ABCG2. These results altogether indicate a direct interaction of both fluorescent dyes with both ABCB1 and ABCG2 in the low micromolar range.

Cytotoxicity of DCV Is Lower Than That of the Ho Dye

Since both DCV and Ho have been suggested to allow sorting of side-population stem cells in flow cytometry for further culturing (13,22), the effects of these dyes on cell survival should be estimated. In order to determine these effects, we measured the concentration-dependent cytotoxicity of both dyes in cultured K562 and A431 cancer cell lines in a 72 h cell survival assays. A431 cells were engineered to stably express a fluorescent marker protein, mOrange, to be used as a viability indicator, which allows in situ a continuous detection of cell survival (Tóth et al., unpublished results).

F2 As shown in Figure 2, both DCV and Ho were toxic in both the parental K562 and A431 cells (Fig. 2A) with low micromolar IC_{50} values (Fig. 2B). Still, DCV toxicity was somewhat lower than Ho toxicity in both cell lines. Expression of the multidrug transporters ABCB1 or ABCG2 significantly reduced the toxicity of both dyes, as expected by the active efflux mediated by these transporters. Interestingly, the protective activity of both ABCB1 and ABCG2 against DCV in these cells was more pronounced than the protection against Ho toxicity. Although the expression levels of the two ABC transporters cannot be directly compared, in these experiments the K562 cells expressing ABCG2 were more protected against DCV than cells expressing ABCB1.

All these data indicate that both DCV and Ho possess strong cytotoxic effects. Still, side-population stem cells, expressing preferentially ABCG2 and showing a reduced dye uptake, may be efficiently sorted without significant effect on cell viability in a DCV concentration range of 1–5 μM.

Comparison of DCV and Ho Uptake in ABCB1- and ABCG2-Expressing Cell Lines as Measured by Flow Cytometry

In the flow cytometry experiments shown in Figure 3, we F3 have examined the dye uptake in intact cells by flow cytometry, and measured ABC transporter expression in parallel by antibody staining to correlate dye uptake and expression levels. For antibody staining of ABCB1 and ABCG2 in intact cells, we have used the cell-surface reactive specific monoclonal antibodies MRK16 and 5D3, respectively (Fig. 3A). In these studies we have also examined, if DCV or Ho is also extruded by the third major multidrug transporter, ABCC1 (MRP1).

In the experiments shown in Figure 3, cellular Ho and DCV fluorescence was measured after 20 min incubation of parental, ABCG2-, or ABCB1-overexpressing K562 (Fig. 3B and 3C) and A431 cells (Fig. 3B and 3D, Supporting Information Fig. S1). The Ho or DCV concentration in the media was 1 μM.

As documented in Figure 3B–3D and Supporting Information Fig. S1, both ABCG2- and ABCB1-expressing cells actively extruded both the DCV and Ho fluorescent dyes, thus, in these cells significantly lower fluorescence was observed than in the parental cell lines. Dye extrusion was inhibited by

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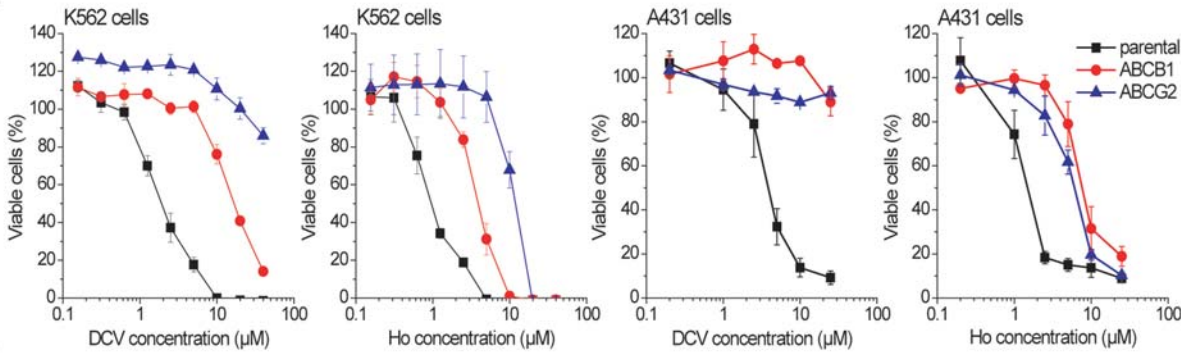
A



B

Cell line	K562			A431		
IC50 (μM)	parental	ABCB1	ABCG2	parental	ABCB1	ABCG2
DyeCycle Violet	1.77 ± 0.08	18.76 ± 5 *	> 40 *	3.52 ± 0.32 *	> 25 *	> 25 *
Hoechst 33342	0.9 ±0.08	3.66 ± 0.22	10.87 ± 0.29	1.18 ± 0.06	6.69 ± 0.29	5.55 ± 0.68

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Figure 2. Cellular toxicity of DCV and Ho in ABCB1- and ABCG2-expressing cell lines. (**A**) Cytotoxic effect of DCV and Ho in K562 and A431 parental, ABCB1- or ABCG2-expressing cells. K562 cell viability was determined by PrestoBlue staining, while in A431 cells expressing mOrange fluorescent protein, viability was measured on the basis of mOrange fluorescence, which is proportional to the cell number. Absorbance or fluorescence of untreated samples considered as 100% for each cell line. The graphs show the average of three independent experiments, error bars indicate SD values. (**B**) Cytotoxicity was determined by IC50 values calculated by dose-response fitting of the Origin software from the viability curves. Significant differences (Student's *t*-test, $P < 0.01$) in the toxicity of Ho and DCV dyes in the same cell lines are indicated by asterisks.

specific transporter inhibitors, and in these cases dye uptake increased to the levels observed in the parental cells.

From these measurements transport activity factors for each transporter and each dye were calculated on the basis of median fluorescence intensities measured in the presence and absence of the specific inhibitor as described in the Materials and Methods section. These data indicate that the activity factors determined either with DCV or with Ho provide a sensitive method for the detection of transporter activity. No substantial DCV or Ho extrusion was found in A431 cells expressing ABCC1 (MRP1) (Fig. 3D).

The exact comparison of the DCV or Ho extrusion activity by the ABCB1 and ABCG2 transporters in these cellular assays is not feasible, as neither the exact amounts of the transporter proteins nor their dye affinity values can be directly determined. Moreover, excitation wavelength options in the FACS instrument used for these experiments are better suited for DCV than for Ho. However, ABCG2 cell lines showed higher DCV extrusion capacity, which is in accordance with the cytotoxicity results above.

Detection of ABCG2 Expression and Function in a High Content Screening (HCS) System at a Single Cell Level

In order to achieve large scale automatic detection and identification of drug-resistant single cells, we developed a real-time, fluorescence-based, automatic HCS assay. In these experiments we have performed in situ HCS measurements to follow DCV or Ho dye uptake in surface-adherent tumor cells at 37°C. In Figure 4, the results of DCV measurements are presented, whereas similar experiments performed with Ho are shown in Supporting Information Figure S2. The imaging data can be properly quantitated and automatized by using the HCS software, even when a large number of the wells are examined in parallel measurements. After completing the dye uptake measurements, we have also performed in situ immunostaining of the same cell populations, in order to examine how dye uptake correlates with transporter expression. Our main goal was to study fluorescent dye uptake in a mixed population of parental and ABCG2 overexpressing cells, as a model of naturally heterogeneous tumor cell populations. F4

In this experiment, we have prepared mixed cell cultures containing 98% of parental and 2% ABCG2-expressing A431 cells, and seeded onto 96 well plates. Following a 16 h incubation, the cells were subjected to 1 μM DCV and fluorescence images were acquired in every 5 min for 150 min. At a given time point a selective ABCG2 inhibitor, Ko143 was added to the samples. At the end of the dye uptake measurement the cells were fixed and in situ immunocytochemistry was performed to visualize ABCG2 expression in the studied cells.

Figure 4A shows the kinetics of DCV uptake in several cells selected from a large dataset. This analysis showed a continuous dye accumulation in the majority of cells, but also identified some cells that showed very low dye uptake, which was significantly accelerated upon the addition of Ko143. Subsequent immunostaining with 5D3 antibody allowed us to identify the ABCG2-expressing cells. Panels 1–3 in Figure 4B show the microscopy images of cellular DCV fluorescence

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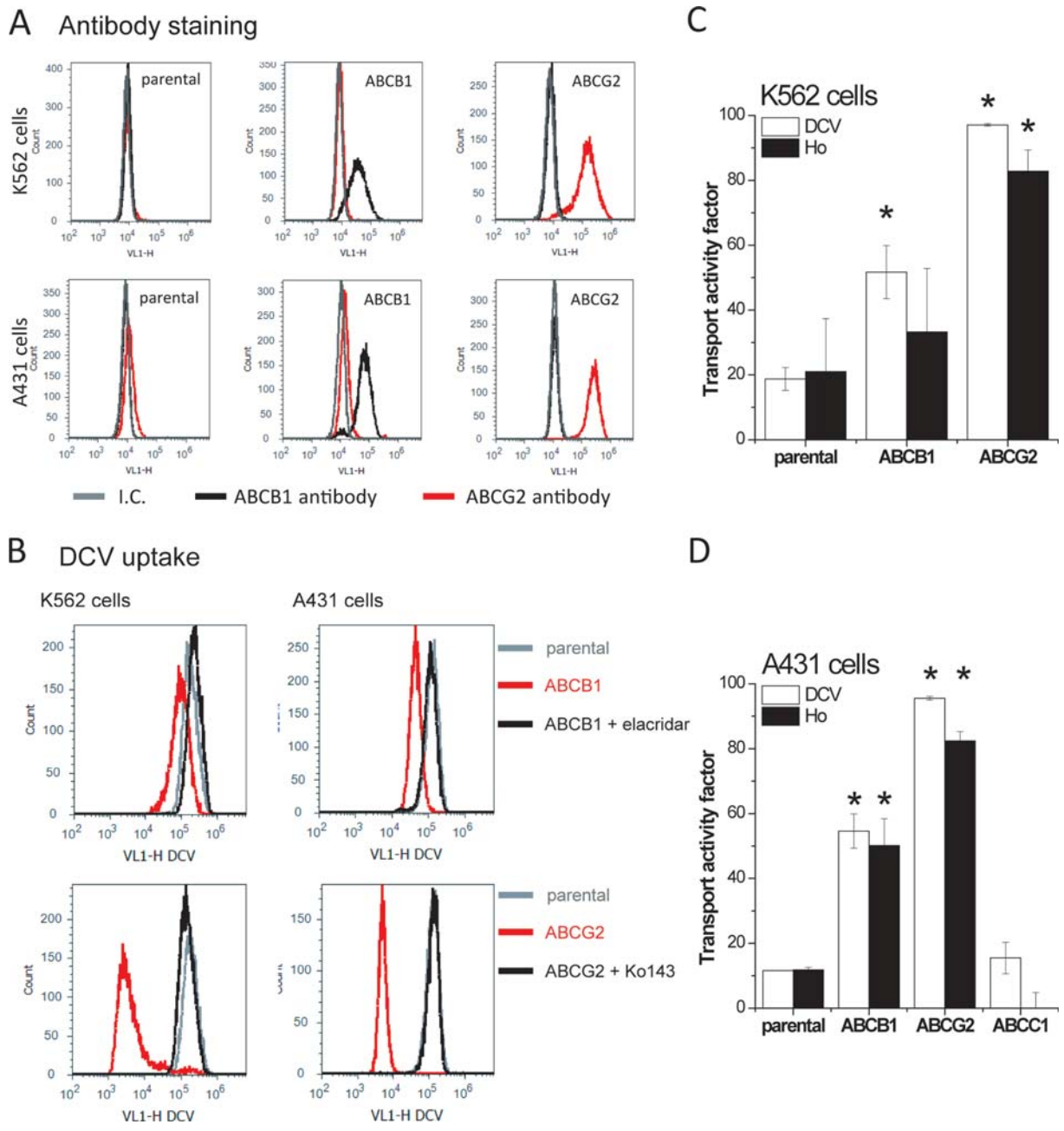

Figure 3. Flow cytometry assessment of immunofluorescence staining and fluorescent dye uptake in ABC transporter expressing cell lines. (**A**) To test the cell surface expression of ABCB1 and ABCG2 in the studied K562 or A431 cell lines, parental, as well as ABCB1- or ABCG2-overexpressing cells were labeled with anti-ABCB1 MRK-16 antibody, anti-ABCG2 5D3 antibody or an isotype control antibody (I.C.), followed by a labeling with a Brilliant Violet 421-conjugated secondary antibody. The fluorescence of the stained samples was assessed by flow cytometry. (**B–D**) DCV and Ho extrusion from K562 or A431 cells by various ABC multidrug transporters. Parental, ABCB1- and ABCG2-overexpressing cells were subjected to DCV (1 μM) or Ho (1 μM) in the absence or the presence of the specific inhibitor of the transporter proteins for 20 min. 5 μM elacridar, 5 μM Ko143, and 100 μM MK571 was used for the inhibition of ABCB1, ABCG2, and ABCC1, respectively. Dye fluorescence in live cells was measured by flow cytometry. (**B**) Representative histograms for DCV uptake in the examined cell lines are shown here, whereas histograms of Ho uptake are presented in Supporting Information Figure S1. Median values of these histograms were determined by Attune Cytometric Software, and the transport activity factors were calculated as described in the Materials and Methods section. The transport activity factors for DCV and Ho in K562 (**C**) and A431 (**D**) cell lines expressing the indicated ABC transporter were determined in at least three independent experiments. Error bars represent SD values; asterisks indicate significant differences ($P < 0.01$).

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obtained at the indicated time points (dye addition was performed at 10 min). Panel 4 show the immunofluorescence image of the same sample stained with 5D3 monoclonal antibody following the DCV uptake measurement. As documented, A431 cells showing slow DCV uptake accurately correspond to the 5D3 labeled, ABCG2-expressing cells.

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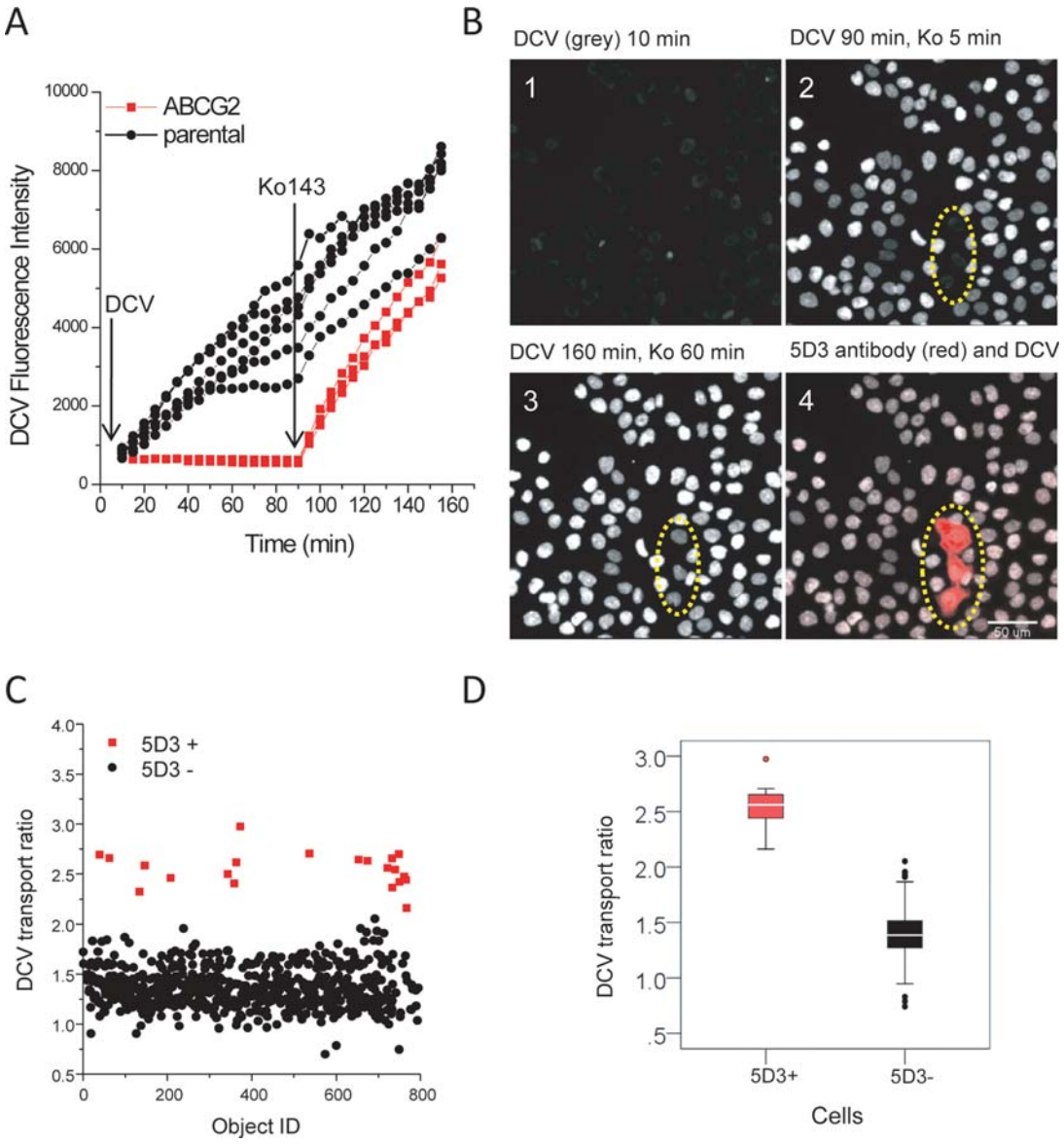

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Figure 4. Detection of ABCG2 expression and function at a single cell level in 96 well plates by using high content screening (HCS) microscopy (**A**) DCV uptake in a mixed population of parental and ABCG2-expressing A431 cells was measured at 37°C by acquiring microscopy images every 5 min. The time courses of average fluorescent intensities of the nuclei of some representative cells in the population are shown. Timing of the additions of DCV (1 μM) and Ko143 (5 μM) are indicated by the arrows. (**B**) Representative microscopy images taken at the indicated time points demonstrate the nuclear accumulation of DCV (grey). Elapsed times after Ko143 indicator are also indicated. Image 4 also illustrates the result of 5D3 antibody labeling (shown in red) preformed after completing the DCV uptake measurement. All images are from the same field of view of the well. ABCG2-expressing cells are marked with a yellow dashed circle. Scale bar in Image 4 represents 50 μm. (**C**) DCV transport ratio in 5D3+ and 5D3− cells. DCV uptake measurement combined with subsequent immunofluorescence staining, as shown in (A) and (B), was performed with a mixed population of parental and ABCG2-expressing A431 cells. DCV transport ratios were calculated from the fluorescence of the individual cell nuclei as described in the Materials and Methods section. The representative field of view contained over 700 cells, which were distinguished by an identification number (Object ID). Cells positive for 5D3 antibody labeling are indicated in red, while cells remained unstained are shown in black. (**D**) Box diagram shows the correlation between DCV transport ratio and the 5D3 positivity of the cells in the same representative site shown in Panel C. Box diagram was prepared by SPSS Software. The difference between the two groups is significant by Student's *t*-test, $P < 0.01$.

Figure 4C and 4D demonstrates a correlation analysis of DCV uptake and 5D3 binding on a large dataset. For this analysis, the results of HCS experiments similar to that demonstrated for selected cells in Figure 4A and 4B were used. On the basis of blue fluorescence of DCV, an identification number (Object ID) was assigned to each cell. Before the addition of Ko143, dye accumulation in the majority of cells nearly plateaued, whereas a minor population exhibited negligible dye uptake (see Figure 4A). Since the fluorescence of the latter was hardly detectable, identification of these nuclei before Ko143 addition was not feasible. Therefore, the transport activity factors cannot be calculated as described previously (21) and

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used for the quantitative analysis of DCV uptake measurements by flow cytometry (Fig. 3C and 3D). To overcome this problem, for quantitative analysis of the HCS measurements, two time points were selected, 15 and 60 min after the addition of Ko143. The fluorescence intensities of the nucleus of each cell were determined at these two time points (F_1 and F_2, respectively). A measure for the dye extrusion activity of the transporter, "DCV transport ratio" was calculated for each cell as F_2/F_1. On the basis of the immunofluorescence staining ABCG2-expressing (5D3+) and non-expressing (5D3−) cell were identified. When the DCV transport ratio values are plotted against the Object ID, marking the 5D3+ cells with red, a strong correlation between DCV transport and ABCG2 expression can be observed (Fig. 4C). Accordingly, 5D3+ cells possess significantly higher DCV transport ratio than 5D3− cells (Fig. 4D).

Discussion

ABC multidrug transporters have a key role in the development of cancer chemotherapy resistance, thus it is of major importance to determine functional transporter expression. Sensitive detection is especially important for targeted therapies and for the identification of highly drug-resistant, potential tumor stem cells. Tumor stem cells are characterized by high tumor renewal potential, while stem cell populations selected and isolated by any methods were found to be heterogeneous both for ABC transporters and stem cell markers (6–8).

It is generally accepted that stem cells can be isolated as a side population after Ho (22–25) or DCV dye (11–13,26–28) staining of living cells. These cells were found to express ABCG2 (25), while in several other cases they express ABCB1 (10,24,29). Still, elevated ABC transporter expression has not been accepted as exclusive stem cell characteristics in many tumor types. In a comprehensive study of ovarian cancer cell lines it was found that after a few passages of ABC transporter-positive side population cells, the transporter-negative population reappeared (26,30). In addition, only a certain fraction of the Ho-selected side population cells showed other stem cell features (30). Despite these uncertainties, side population identification is widely used as a first step in stem cell isolation, and proper conditions as well as sensitive methods can increase the selectivity for better defined stem cell populations (8,10,24,25,31).

In this work we analyzed the dye interactions with the key ABC multidrug transporters and developed a HCS imaging-based, combined functional and protein expression analysis for the sensitive detection of ABC multidrug transporters in individual cells of heterogeneous cell populations. The DCV dye resembles the Ho dye in several aspects, but here we document that DCV is more suitable for various instruments in terms of fluorescence parameters, and better applicable in the ABC transporter assays for several reasons.

Direct transporter-dye interaction was examined by measuring ABC-ATPase activity in a membrane vesicle assay. Both DCV and Ho dye inhibited ATPase activity of ABCG2 and ABCB1 in a concentration-dependent manner, suggesting that these dyes are slowly transported substrates. In the ABCG2-ATPase assay the IC_{50} value of DCV was slightly higher than that of Ho, which may indicate a different, probably higher transport rate for DCV.

Although both DCV and Ho, as DNA binding dyes, are potentially toxic compounds, earlier studies showed that, at least in short term experiments, none of these dyes caused significant reduction in cell viability or changes in gene expression (10). Here we demonstrate in two cancer cell lines (K562 and A431) that both dyes are toxic in micromolar concentrations. Still, the effective toxic concentration of DCV is significantly higher than that of Ho, and the difference is even more pronounced in cell lines expressing the ABCB1 or ABCG2 transporters. Thus, DCV is preferred over Ho regarding their toxicity.

Various flow cytometry assays have already been developed for these clinically important multidrug transporters by using transporter positive and negative cell line pairs and fluorescent indicator substrates (32,33). Our results here show that DCV is an excellent indicator in this regard and we assume that it can also be applied for screening transporter inhibitors.

An important point in this work is the development of a new HCS application by using DCV for following ABC transporter activity in single cells. High throughput flow cytometry (32,33) and high content imaging (34,35) are both applicable to screen for substrates and inhibitors of ABC transporters, but HCS has the key advantage to allow these studies in situ, in surface-attached living cells. In addition, both transporter activity assay and immunostaining can be performed in a mixed cell population.

As a model for heterogeneous cancer cell populations, we used the parental and ABCG2-overexpressing A431 cells. Here we document that after a careful optimization of DCV concentrations and timing of the dye uptake experiments, DCV can be used to identify even a low number of ABCG2-positive cells in a large non-expressing population. DCV toxicity was negligible in the applied concentration and time frame, and the selection of transporter-positive cells based on DCV staining closely correlated with ABCG2-specific antibody staining. Thus, side-population selection by DCV dye uptake measurements in HCS microscopy can be combined with parallel antibody staining for various potential stem cell markers, providing a great advantage in characterizing the low number of cancer stem cells.

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