A High-Content Assay Strategy for the Identification and Profiling of ABCG2 Modulators in Live Cells

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ABSTRACT

ABCG2 is a member of the ATP-binding cassette (ABC) family of transporters, the overexpression of which has been implicated in resistance to various chemotherapeutic agents. Though a number of cell-based assays to screen for inhibitors have been reported, they do not provide a content-rich platform to discriminate toxic and autofluorescent compounds. To fill this gap, we developed a live high-content cell-based assay to identify inhibitors of ABCG2-mediated transport and, at the same time, assess their cytotoxic effect and potential optical interference. We used a pair of isogenic U87MG human glioblastoma cell lines, with one stably overexpressing the ABCG2 transporter. JC-1 (J-aggregate-forming lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol carbocyanine iodide) was selected as the optimal reporter substrate for ABCG2 activity, and the resulting assay was characterized by a Z' value of 0.50 and a signal-to-noise (S/N) ratio of 14 in a pilot screen of ~7,000 diverse chemicals. The screen led to the identification of 64 unique nontoxic positives, yielding an initial hit rate of 1%, with 58 of them being confirmed activity. In addition, treatment with two selected confirmed positives suppressed the side population of U87MG-ABCG2 cells that was able to efflux the Hoechst dye as measured by flow cytometry, confirming that they constitute potent new ABCG2 transporter inhibitors. Our results demonstrate that our live cell and content-rich platform enables the rapid identification and profiling of ABCG2 modulators, and this new strategy opens the door to the discovery of compounds targeting the expression and/or trafficking of ABC transporters as an alternative to functional inhibitors that failed in the clinic.

INTRODUCTION

Multidrug resistance (MDR) constitutes the main mechanism that is responsible for the resistance of cancer cells to standard therapy. MDR is often acquired by overexpression of ATP-binding cassette (ABC) transporters, a superfamily of transmembrane pumps with broad specificity for various chemical substrates. The three ABC transporters most overexpressed in cancer are ABCB1, ABCC1, and ABCG2,¹ and the overexpression of ABC transporters allows MDR cells to become resistant to multiple drugs through increased efflux from the cell. Overexpression of ABCG2, the breast cancer resistance protein (BCRP), has been found to be associated with resistance to a wide range of different anticancer agents, including mitoxantrone, camptothecins, anthracyclines, flavopiridol, and antifolates.² ABCG2 is often expressed in stem cell populations, and stem cells can be isolated by fluorescence-activated cell sorting (FACS) by sorting the cell population that exhibits low levels of Hoechst staining, as ABC transporters have the ability to exclude dyes in addition to drugs.³ Due to this property, stem cells are often referred to as the “side population.”

In gliomas, it was found that only the ABCG2 pump is overexpressed, in agreement with literature establishing ABCG2 as the main stem cell-associated ABC transporter.⁴ In addition, ABCG2 constitutes a major contributor to the blood-brain barrier, restricting drug distribution and delivery to brain cells.⁵⁻⁷ Therefore, the identification of compounds that are able to modulate this transporter could potentially improve the efficiency of a variety of chemotherapeutic agents for cancer, and for gliomas in particular. Despite significant efforts, suitable ABCG2 inhibitors are still lacking. Several *in vitro* assays have been established for the identification of new ABCG2 modulators, such as drug-efflux activity using FACS,⁸⁻¹² transport assays measuring the net flux across the monolayer,¹³ bioluminescence imaging,¹⁴ and ATPase assays.¹⁵ All these assays measure only one parameter and provide hits based on a single criterion: the ABCG2 function, as measured by the efflux of a fluorescent substrate. Such assays cannot discriminate between inhibitors competing with the site-specific substrate and those compounds

ABBREVIATIONS: ABC transporter, ATP-binding cassette transporter; BCRP, breast cancer resistance protein; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; INCA2000, IN CELL Analyzer 2000; INCA3000, IN CELL Analyzer 3000; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FTC, fumitremorgin C; HC, high control; LC, low control; IgG, immunoglobulin G; JC-1, J-aggregate-forming lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol carbocyanine iodide; MDR, multidrug resistance; PBS, phosphate-buffered saline.

affecting the expression and trafficking of ABCG2. Various inhibitory molecules have been identified,¹⁶ and clinical trials with the third-generation MDR inhibitors are still ongoing; however, results are not promising,¹⁷ suggesting the need for a new approach.

An alternative strategy to overcome ABCG2-mediated MDR is the development of modulators that specifically target the expression and trafficking of ABCG2. To date, little is known about the intracellular distribution of the ABCG2 transporter and the mechanisms modulating its localization and expression. It became evident recently that in addition to cellular membrane localization, transporters can be localized intracellularly in vesicles.¹⁸ Therefore, studying the intracellular localization of drug transporters and the modulation of their cellular trafficking could be crucial to understanding the process of cellular drug uptake and retention. Importantly, this approach could yield new drug candidates with an alternative mechanism of action compared with compounds strictly targeting the function of these transporters. We have previously shown that such an approach can be successful in identifying compounds modulating epidermal growth factor receptor (EGFR) activation by a mechanism of action that is distinct from targeting the tyrosine kinase activity of the receptor.¹⁹ However, to identify and characterize such modulators of the expression and trafficking of the ABCG2 transporter, a high-content screening approach that would enable multiple readouts from the same well is needed.

In this study, we establish for the first time a screening approach for ABCG2 modulators that takes advantage of multiplexed readouts allowed by automated microscopy and image analysis, for the simultaneous identification of inhibitors of ABCG2 transporter and the characterization of cytotoxic and autofluorescent compounds, opening the door to the characterization of those compounds modulating ABCG2 expression and trafficking.

MATERIALS AND METHODS

Reagents

The JC-1 (J-aggregate-forming lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol carbocyanine iodide) substrate, Dulbecco's modified Eagle's medium (DMEM), Hank's buffered salt solution (HBSS), penicillin, streptomycin, puromycin, phosphate-buffered saline without Mg^{2+} and Ca^{2+} (PBS), normal goat serum, phalloidin-Alexa Fluor 488, and goat antimouse immunoglobulin G (IgG) antibody conjugated with Alexa Fluor 568 were purchased from Life Technologies. Neural Stem Cell basal medium was purchased from STEMCELL Technologies. Mouse monoclonal anti-BCRP, clone 5D3 antibodies were purchased from Millipore. Bovine serum albumin, Triton X-100, Tween 20, and nifedipine hydrochloride were purchased from Sigma-Aldrich.

Cell Lines and Tissue Culture

The human glioblastomacell lines U87MG, HTB14, and HTB15 were purchased from the American Type Culture Collection. The U87MG-ABCG2 cells were generated by stable transfection with hABCG2 as previously described.²⁰ U87MG, HTB14, and HTB15 cells were cultured in DMEM supplemented with 10% heat-inactivated

fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 µg/mL). U87MG-ABCG2 cells were cultured in the same medium with the addition of puromycin (4 µg/mL) as the selection agent.

Automated Image Acquisition and Analysis

For the Hoechst and JC-1 efflux assays, whole-well images were acquired using the IN Cell Analyzer 2000 (INCA2000) automated epifluorescence microscope (GE Healthcare) at 4× magnification (0.20 numerical aperture). This lamp-based microscope equipped with a long-life wide field illumination source captures transmitted light images using a CoolSNAP CCD camera (Photometrics). For the Hoechst efflux assay, images were acquired using 350/50 nm excitation and 455/50 nm emission filters with a 0.1 s exposure time. For the JC-1 efflux assay, images were acquired using 490/20 nm excitation and 525/36 nm emission filters with a 0.6 s exposure time, and using 350/50 nm excitation and 455/50 nm emission filters with a 0.1 s exposure time to image nuclei on the Hoechst channel. An automated image analysis was conducted on raw unprocessed images with IN Cell Developer 1.7 software (GE Healthcare), using custom-developed analysis protocols that rely on built-in object-based segmentation algorithms with set parameters for object size and sensitivity of detection. For the sake of consistency, the same protocol with the same exact parameters was applied to all images and for the two replicate runs. The custom protocol developed for the efflux readout segments objects on the JC-1 channel and quantifies intensity per object; data are reported as the sum of fluorescence intensity for all objects. The custom protocol developed for the nuclei count readout identifies nuclei by object segmentation on the Hoechst channel and quantifies the total nuclei count.

Chemical Libraries

The library screened combines 6,912 chemicals obtained from MicroSource, Prestwick, Tocris, and other commercial sources as previously described.¹⁹ The MicroSource Library contains 2,000 known drugs, natural products, and other bioactive components, such as enzyme inhibitors, receptor blockers, membrane-active compounds, and cellular toxins. The Prestwick Chemical Library is a unique collection of 1,119 marketed drugs, selected for their high chemical and pharmacological diversity, as well as known bioavailability and safety in humans. Tocris library represents a unique and diverse collection of high purity compounds with known activity in kinases, ion channels, nuclear receptors, and transporter assays.

High-Resolution Imaging of ABCG2 Expression in Chambered Coverglass

High-resolution imaging of U87MG and U87MG-ABCG2 cells seeded on chambered coverglass at 60,000 cells/chamber in 400 µL medium and incubated with 10 µM inhibitor for 16 h was conducted on a Zeiss Axioplan 2 Imaging Widefield Microscope at 100× objective magnification after cell fixation and staining as previously described. Immunostaining of ABCG2 was conducted using as a primary antibody the monoclonal anti-BCRP clone 5D3, recognizing an external epitope of ABCG2 at a final concentration of 2 µg/mL

(1:300 stock dilution) in PBS for 1 h, followed by three washes with blocking buffer. The secondary antibody goat anti-mouse IgG conjugated to Alexa Fluor 568 was added for 1 h at room temperature, followed by three PBS washes.

Assay Development and Optimization

We sought to develop an ABCG2 efflux assay in live cells, relying on the difference in intracellular fluorescence intensity of cells that actively pump out the fluorescent substrate and cells that accumulate the substrate. For this purpose, we measured the accumulation of the two fluorescent substrates, Hoechst and JC-1, in the U87MG-ABCG2 cell line and its isogenic counterpart U87MG as a control in a 384-well format (Corning #3985). To evaluate the stability of the signal over time, U87MG and U87MG-ABCG2 cell suspensions were dispensed into 384-well assay plates at a density of 3,500 cells/well in a 45 μ L medium using an automated Multidrop 384 dispenser (Thermo Electron Corp.). At 20 min and 60 min post-treatment for both substrates, and up to 16 h, 18 h, and 22 h post-treatment for JC-1, the assay plates were imaged using the INCA 2000 and automated image analysis was conducted as previously described for the efflux readout.

To optimize cell density, cells were seeded at densities of 1,750, 3,500, 5,000, 7,000, and 10,000 cells/well. At 16 h postseeding, plates were imaged using the INCA 2000, followed by automated image analysis for the efflux readout.

To validate the optimal conditions selected for the assay, U87MG and U87MG-ABCG2 cell suspensions were dispensed using Multidrop at a density of 3,500 cells/well in a 45 μ L medium into a 384-well assay plate with 5 μ L preplated high controls (HCs; 250 μ M nifedipine in 10% v/v dimethyl sulfoxide [DMSO]) and low controls (LCs; 10% v/v DMSO) and incubated for 16 h, followed by imaging and image analysis to extract the efflux readout.

Dose-Response Studies

For validation of the assay conditions and for confirmation of screen positives, compound potency was assessed in dose response using 12 doubling dilutions in duplicate with a 100 μ M and 10 μ M compound concentration as the upper limit. Controls consisted of 25 μ M nifedipine in 1% (v/v) DMSO (HC) and 1% (v/v) DMSO (LC) final concentrations. Control and compound dilutions were made in an intermediate 384-well polypropylene plate (ABgene, Thermo Fisher Scientific), and 5 μ L was transferred to the assay plates with a custom-designed 384 head on a PP-384-M Personal Pipettor (Apricot Designs). Cells were then seeded at 3,500 cells/well in 45 μ L of medium containing 5% (v/v) FBS and 150 nM JC-1. A lower amount of FBS compared with tissue culture conditions (10% v/v) was used to reduce background fluorescence. After 16 h of incubation, the assay plates were imaged and analyzed as previously described.

After an automated image analysis, dose-response data files were uploaded onto the HTS Core Screening Data Management System for curve fitting and IC₅₀ calculation. Curves presented in this article were fitted as a logistic 4-parameter sigmoid using SigmaPlot 9.0 (Systat Software, Inc.). Inhibition of JC-1 accumulation (JC-1 efflux readout) and cell proliferation (nuclei count readout) were expressed as percentage compared with HCs and LCs, and defined as % inhibition = (HC average – read value)/(HC average – LC average) $\times$ 100.

Side Population Analysis

U87MG and U87MG-ABCG2 cells were treated with compounds for 16 h at 37°C. Cells were then trypsinized and resuspended at 2E6 cells/mL in Neural Stem Cell basal medium (Stem Cell Technology) and incubated for 90 min at 37°C with 5 mg/mL Hoechst 33342. After staining, cells were washed with ice-cold PBS and resuspended in HBSS. Hoechst dye was excited at 407 nm by trigon violet laser, and dual wavelengths were read using 450/40 nm and 695/40 nm filters. Dead cells were excluded by gating on forward and side scatter and by eliminating propidium iodide-positive population. The resulting data were analyzed by FlowJo.

JC-1 Efflux Screening Assay for Inhibitors of ABCG2 in 384-Well Format

Screening of the chemical library of 6,912 compounds (described in the *Chemical Libraries* section) was conducted in duplicate using the previously optimized assay conditions for the live JC-1 efflux assay (Table 1). Compounds were preplated in an intermediate 384-

Table 1. Workflow of the Optimized Live Cell Assay for JC-1 Efflux in U87MG-ABCG2 Cells

Step	Parameter	Value	Description
1	Library compounds	5 μ L	100 μ M in 10% (v/v) DMSO
2	Low control	5 μ L	10% (v/v) DMSO
3	High control	5 μ L	250 μ M nifedipine in 10% (v/v) DMSO
4	Cell plating	45 μ L	3,500 U87MG-ABCG2 cells in DMEM media with 5% FBS and 150 nM JC-1
5	Incubation time	16 h	37°C, 5% CO ₂ in Stericult automated temperature controlled incubator
6	Assay readout	488 nm/535 nm (ex/em)	Imaging using IN Cell Analyzer 2000 automated microscope
7	Image analysis		Analysis using Developer Toolbox 1.7 software

Step Notes

- 1–3. Dispensing on the PP-384-M Personal Pipettor using a custom 384 head.
4. Cells prepared in the media premixed with JC-1 and dispensed into a 384-well assay plate with Multidrop 384.
6. Live cell imaging performed on a semi-automated platform.

DMSO, dimethyl sulfoxide; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol carbocyanine iodide; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.

well poly-propylene plate at 100 μ M in 10% (v/v) DMSO, and 5 μ L were transferred to the assay plates using Personal Pipettor for a final assay concentration of 10 μ M in 1% (v/v) DMSO. Controls present in each assay plate consisted of 25 μ M nicardipine in 1% (v/v) DMSO as HCs and 1% (v/v) DMSO as LCs (final concentrations). U87MG-ABCG2 cells pre-mixed with 150 nM JC-1 were seeded into wells at 3,500 cells/well in 45 μ L medium with 5% FBS (v/v) using Multidrop. After 16 h of incubation, the assay plates were imaged on the INCA2000 and analyzed with IN Cell Developer 1.7 software for JC-1 efflux readout. After live JC-1 efflux readout, screening plates were fixed and stained using the Biotek washer for aspirations and Multidrop for liquid dispensing. Plates were fixed with 4% (v/v) paraformaldehyde for 20 min, washed twice with PBS, and nuclei were stained with a solution of 10 μ M Hoechst in PBS for 10 min. After two washes in PBS, plates were imaged and analyzed for nuclei count as described earlier.

RESULTS

Assay Development and Optimization

We sought to develop a cell-based high-content assay that would not only enable identification of the inhibitors of the efflux activity of ABCG2, but also discriminate cytotoxic and autofluorescent compounds. For this purpose, we took advantage of an isogenic pair of cell lines, the ABCG2-overexpressing cell line U87MG-ABCG2 and its parental counterpart U87MG to measure ABCG2 efflux in live cells. The assay principle lies in the reduced accumulation of an ABCG2-specific fluorescent substrate in U87MG-ABCG2 cells as compared with the parental U87MG cells, due to its active efflux by the ABCG2 transporter in U87MG-ABCG2 cells (*Fig. 1*). To confirm overexpression of ABCG2 in U87MG-ABCG2 cells compared with the parental U87MG cells, we visualized ABCG2 expression in U87MG and U87MG-ABCG2 cells by high-resolution imaging after ABCG2 immunostaining. As expected, no staining could be observed for U87MG cells, while the intensity of staining was strong for U87MG-ABCG2 cells, confined to the cell membrane and also distributed throughout the cell (*Fig. 2*). This result validates our cell model.

We first investigated the use of the Hoechst stain for nuclei as the fluorescent substrate to be used in our assay. As expected, Hoechst total fluorescence intensity was significantly higher in U87MG than in U87MG-ABCG2 cells after 20 min of incubation, by approximately four-fold. However, after 60 min, the difference in signal between the two cell lines decreased significantly, due to Hoechst accumulation in U87MG-ABCG2 cells (*Fig. 3A*). This result can be explained by tropism of the Hoechst dye for the nucleus, resulting in DNA intercalation and subsequent absence of the dye in the cytoplasm, preventing its efflux (*Fig. 1*). This phenomenon makes Hoechst unsuitable for the assay. For this reason, we investigated the use of the JC-1 dye as an alternative fluorescent ABCG2 substrate.^{9,12} In contrast to Hoechst, the significant difference in JC-1 intracellular accumulation between U87MG and U87MG-ABCG2 (three- to eight-fold) remains constant for $\sim$ 22 h (*Fig. 3B–D*), thus being more amenable to our assay and automated screening. As expected, in the HTB14 and HTB15 human glioblastoma cell lines that do not express

Fig. 1. Schematic of Hoechst and JC-1 dyes intracellular accumulation dynamics as probes for ABCG2-mediated efflux. Hoechst and JC-1 substrates cross the membrane of U87MG and U87MG-ABCG2 cells by passive diffusion and accumulate in parental U87MG cells, producing high intracellular fluorescence intensity. In ABCG2-expressing cells, a fraction of the Hoechst dye is pumped out of the cell while the other translocates to the nucleus and intercalates with DNA. Over time, more Hoechst accumulates in the nucleus, increasing fluorescence intensity in ABCG2-expressing cells up to the level of parental U87MG cells. In contrast, JC-1 resides in the cytoplasm and is efficiently pumped out by the ABCG2 transporter. This process reaches equilibrium and maintains low intracellular fluorescence intensity over time, with JC-1 thus constituting a better probe for the assay. JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol carbocyanine iodide.

ABCG2, JC-1 diffused inside the cells, and in the absence of efflux, accumulated in the cytoplasm, leading to high intracellular fluorescence intensity that was similar to U87MG cells as compared with U87MG-ABCG2 cells (*Fig. 3E*).

To optimize cell density for our assay, we tested in parallel five cell seeding densities between 1,750 and 10,000 cells/well in a 384-well format. After 16 h of incubation, clumping patterns were observed for 5,000 cells seeded per well and above; this cell overlap interfered with an automated image analysis for which single cell identification is necessary for accurate quantification (data not shown). As a consequence, we selected 3,500 cells/well as the optimal cell seeding density for the assay, as it allowed for the largest accurate cell count achievable.

As a control for the assay, we evaluated the known ABCG2 pump inhibitor nicardipine.²¹ As expected, treatment of U87MG-ABCG2 cells with 25 μ M nicardipine fully restored JC-1 intracellular fluorescence intensity levels to those observed with U87MG cells that were unable to efflux JC-1 (*Fig. 4A, B*); this result indicates that

Fig. 2. High-resolution imaging of ABCG2 expression in U87MG and U87MG-ABCG2. Representative images acquired using a Zeiss Axioplan 2 widefield microscope at 100 $\times$ magnification of U87MG and U87MG-ABCG2 cells immunostained for ABCG2 (red) with phalloidin-AF488 stained actin (green) and Hoechst-stained nuclei (blue).

ABCG2 pumps in U87MG-ABCG2 cells are fully inhibited by nicardipine at that concentration and that we can measure inhibition of ABCG2 efflux in these cells using our assay.

As a proof of concept, we assessed the dose response of nicardipine and of the potent ABCG2 pump inhibitor Fumitremorgin C (FTC)^{22,23} using the newly optimized assay conditions. The dose-response curves obtained using our assay allowed us to calculate the IC₅₀ for these compounds, $6.40 \pm 1.2 \mu\text{M}$ for nicardipine (Fig. 4C) and $0.60 \pm 0.07 \mu\text{M}$ for FTC (Fig. 4D). Those values are similar to the published values of $4.8 \pm 1.3 \mu\text{M}$ for nicardipine²¹ and $0.40 \pm 0.10 \mu\text{M}$ for FTC,²⁴ demonstrating that the optimized conditions which we have selected for our assay (Table 1) allow us to accurately measure and quantify ABCG2 efflux in live cells and in a miniaturized format that is compatible with HTS. Based on the dose-response curve of nicardipine in the JC-1 efflux assay, we selected 25 μM nicardipine in 1% (v/v) DMSO as the HC for our assay, as it leads to full JC-1 efflux inhibition (Fig. 4B, C). The LC consists of 1% (v/v) DMSO as the compound carrier.

Chemical Screen for Modulators of ABCG2

Having successfully developed and optimized a cell-based assay for ABCG2 efflux relying on the JC-1 probe, we sought to validate a platform that would allow us to identify inhibitors of ABCG2 efflux activity, while discriminating cytotoxic and autofluorescent compounds. For this purpose, we screened a chemical library of 6,912 known bioactives, U.S. Food and Drug Administration–approved drugs, and experimental substances for inhibitors of ABCG2 efflux in U87MG-ABCG2 cells using our optimized assay, followed by confirmation of hits.

The chemical library was plated at a compound screening concentration of 10 μM in 1% (v/v) DMSO in twenty 384-well microtiter plates, and the screen was performed in duplicate, yielding two sets of data: set 1 and set 2. It should be noted that while day 2 of the screen

was ongoing, the light source on the INCA 2000 was malfunctioning at times, leaving us unable to rescue the screen because it is a live cell imaging assay. We evaluated the robustness of the screen by analyzing the statistical performance of HC and LC wells present in each screening plate (Fig. 5A). Control values for JC-1 efflux as measured by the sum of object intensity were consistent between the two sets of data and with assay development data, with an overall average value of 15,067 for HC wells and 1,095 for LC wells (Fig. 5B). Both replicate screens yielded a large S/N ratio between HC and LC wells, respectively 9- and 24-fold for set 1 and set 2, which along with a low variability of HC wells (coefficient of variation [CV] of 9% for both sets) and acceptable variability for LC wells yielded a well-defined signal window defined by Z' values of 0.47 for set 1 and 0.67 for set 2 (Fig. 5B). The relatively high CV values for LC wells of up to 78% are mainly due to differences between small numbers of all data presented without elimination of outliers, and, in reality, do not significantly affect the performance of the assay that is characterized by a S/N ratio >10 and an overall Z' value >0.5, indicative of the good robustness of the assay during the entire screen.²⁵

Each compound was screened in duplicate to assess the reproducibility of the assay during the screen. The scatter plot of the percentage inhibition of JC-1 efflux induced by each compound in each set of data shows an overall good correlation between the two sets of data ($R^2 = 0.74$; Fig. 5C), despite a shift toward higher percentage inhibition in data set 2. This is likely due to the encountered issues with the microscope light source during image acquisition of the second replicate of data, highlighting one of the many challenges in performing live cell imaging. Not surprising was the identification of compounds inducing an apparent high percentage inhibition (up to 400%), constituting likely autofluorescent compounds, and evaluation of optical interference is taken into account in our confirmatory workflow described later in this article. When plotting the percentage inhibition in nuclei count induced by each compound and

Fig. 3. Kinetics of Hoechst and JC-1 probe accumulation in U87MG and U87MG-ABCG2 cells. Quantification of Hoechst accumulation in U87MG-ABCG2 cells over 60 min (**A**) and of the JC-1 probe (**B**), maintaining a large signal window between probe accumulation in U87MG and U87MG-ABCG2 cells for ~22 h. The data presented are means $\pm$ standard deviation of 192 replicate wells. Representative images of U87MG and U87MG-ABCG2 cells at 20 and 60 min post-probe addition for quantification of Hoechst (**C**) and JC-1 (**D**) probe accumulation. Comparative panel of representative images for U87MG, U87MG-ABCG2, HTB14, and HTB15 cells at 60 min post JC-1 probe addition (**E**). Images were acquired with the INCA2000 automated microscope in the blue channel for Hoechst and in the green channel for JC-1 at 20 $\times$ objective magnification.

Fig. 4. Inhibition of JC-1 efflux. **(A)** Quantification of JC-1 probe accumulation in U87MG compared with U87MG-ABCG2 cells treated with 1% (v/v) DMSO as a control and 25 μM nicardipine in 1% (v/v) DMSO. The data presented are means $\pm$ standard deviation of 32 replicate wells. **(B)** Representative images of U87MG-ABCG2 cells seeded with JC-1 probe in the presence of DMSO control or 25 μM nicardipine at 16 h postseeding. Overlay of automated image analysis for quantification of JC-1 efflux. **(C)** Dose-response curve in the JC-1 efflux assay of U87MG-ABCG2 cells treated with nicardipine, yielding a calculated IC_{50} of $6.40 \pm 1.20 \mu\text{M}$. The data presented are means $\pm$ standard deviation estimated from duplicate wells. **(D)** Dose-response curve in the JC-1 efflux assay of U87MG-ABCG2 cells treated with Fumitremorgin C (FTC), yielding a calculated IC_{50} of $0.60 \pm 0.07 \mu\text{M}$. The data presented are means $\pm$ standard deviation estimated from duplicate wells.

for each set of data as a scatter plot, we found that most compounds had little effect on cell count, with the cloud of compounds centered between 0% and 50% inhibition (Fig. 5D). This result is consistent with our earlier screens performed over shorter incubation times.¹⁹ The linear distribution of the percentage inhibition in nuclei count readout ($R^2=0.78$) and the presence of few outliers further demonstrate the good reproducibility of our assay during screening. Alto-

gether, our results demonstrate that our assay is compatible with HTS, being associated with good robustness and reproducibility during this screen, ensuring accurate and consistent positive selection across screening plates.

To assess whether our ABCG2 efflux assay was able to identify inhibitors of the ABCG2 pump in live cells, we plotted the average percentage inhibition of JC-1 efflux induced by each compound

against the average percentage inhibition induced in nuclei count, and we highlighted known ABCG2 inhibitors in the resulting scatter plot. Importantly, seven known ABCG2 inhibitors clustered in this graph as inducing a high percentage inhibition in JC-1 efflux while inducing a low percentage inhibition in nuclei count (*Fig. 5E* and *Table 2*). This result validates our strategy, in that our ABCG2 efflux assay can identify ABCG2 inhibitors in live cells among a library of chemicals screened in 384-well microplates.

For positive selection, we defined our thresholds based on overall data distribution and on the performance of the known ABCG2 efflux inhibitors present in the library. We observed that all known ABCG2 inhibitors induced >70% inhibition in the absence of any significant effect on the cell number, with <20% reduction in nuclei count (*Fig. 5E*). For this reason, we selected a threshold of 70% inhibition of JC-1 efflux or greater and a 20% inhibition of nuclei count or lower to identify nontoxic inhibitors of ABCG2 in our screen. Based on this threshold, we selected 80 positives consisting of 77 unique compounds (*Fig. 5F*).

Dose-Response Studies

To confirm the selected screen positives, we tested dose response in 65 resupplied positives (64 unique compounds) available from the suppliers that have passed quality control and purity standards from vendors. Resupplied positives were tested in our live cell ABCG2 efflux assay at 12 doubling dilutions ranging from 5 nM to 10 μ M using U87MG-ABCG2 cells and three other glioblastoma cell lines that do not express ABCG2. We also included as a control dose-response plates with U87MG-ABCG2 cells but no added JC-1. This allowed us to sort out the identified inhibitors into three categories: (1) true inhibitor; (2) autofluorescent true inhibitor; and (3) autofluorescent compound. In the presence of a true inhibitor such as α -mangostin, no fluorescence is detected in the absence of JC-1 probe (*Fig. 6A*) and a dose-dependent increase in JC-1 intracellular accumulation is observed (*Fig. 6B*). For compounds such as YM 90709, intracellular background fluorescence is observed in cells in the absence of JC-1, indicating that the compound is autofluorescent

Table 2. Known ABCG2 Modulators Identified as Positives in the Screen

Compound name	HTS %I JC-1 efflux ^a	HTS %I nuclei count ^b	JC-1 efflux IC ₅₀ (μ M) ^c
Lapatinib ²⁶	144	12	5.8 $\pm$ 0.8
Imatinib ²⁷	94	13	7.0 $\pm$ 2.9
Dipyridamole ²¹	86	5	2.0 $\pm$ 0.8
Acacetin ²⁸	78	3	1.5 $\pm$ 1.6
Apigenin ²⁹	92	21	17 $\pm$ 2.2
Bromocriptine mesylate ³⁰	100	11	5.7 $\pm$ 0.01
Ivermectin ^{13,31}	70	-2	12 $\pm$ 0.3

^aPerformance during the screen in the JC-1 efflux readout.

^bPerformance during the screen in the nuclei count readout.

^cCalculated IC₅₀ in the JC-1 efflux readout.

HTS, high-throughput screening; %I, percentage inhibition.

(*Fig. 6A*). This background autofluorescence is quantified and apparent in the dose-response curve for this compound in the form of a higher baseline; however, an increase in probe accumulation is observed when increasing compound concentration, which is not the case in the absence of the probe (*Fig. 6C*); this indicates that the compound is a fluorescent true inhibitor. Finally, some compounds, such as PHA 665752, induce a dose-dependent increase in fluorescence even in the absence of the probe, demonstrating that they likely do not constitute true inhibitors and rather are autofluorescent compounds (*Fig. 6A, D*).

With 58 out of 65 resupplied positives inducing potent inhibition in the assay, the confirmation rate was nearly 90%, which was indicative of accurate positive selection during screening. The 10%

Fig. 5. Robustness and reproducibility assessment of the JC-1 efflux assay during screening and positive selection. A library of 6,912 compounds was screened in duplicate (set 1 and set 2) and plated in a total of forty 384-well plates. Each screening plate included high control wells (HC) containing 1% (v/v) DMSO and low control wells (LC) containing 25 μ M nicardipine in 1% (v/v) DMSO to assess the robustness of the assay during screening. **(A)** Scatter plot analysis of the sum of JC-1 intracellular intensity and frequency distribution of HC and LC in set 1 and set 2, replicate sets of data, and overall. **(B)** Summary table of statistical analysis of controls during screening. The average sum of JC-1 intracellular intensity, standard deviation, CV for HC and LC, signal-to-noise ratio (S/N), and calculated Z' value are presented. For data sets 1 and 2, all 320 HC and 320 LC wells were used to calculate the Z' value, and the 640 HC and 640 LC wells were used for the overall Z' value calculation. The relatively high CV values for LC wells of up to 78% did not significantly affect the performance of the assay that was characterized by a S/N ratio >10, as demonstrated by the overall Z' value >0.5. **(C)** The percentage inhibition for JC-1 efflux induced by each compound and in each set of data is plotted as a scatter plot ($R^2=0.74$), demonstrating the overall good reproducibility of the live efflux readout during the screen, in spite of issues with the INCA2000 light source that led to a shift toward higher percentage inhibition in data set 2. **(D)** The nuclei count induced by each compound and for each data set is plotted as a scatter plot ($R^2=0.78$), demonstrating the reproducibility of the nuclei count readout during the pilot screen. **(E)** For positive selection, the average percentage inhibition for JC-1 efflux for each compound and for both replicate data sets is plotted against the average percentage inhibition in the nuclei count of each compound. Seven reported ABCG2 inhibitors are clustered in the scatter plot, inducing a high percentage inhibition in JC-1 efflux and a low percentage inhibition in nuclei count. **(F)** 80 initial positives (77 unique compounds) are selected as those compounds inducing >70% inhibition in JC-1 efflux and <20% inhibition in nuclei count.

Fig. 6. Confirmation in dose response of initial positives in the JC-1 efflux assay and segregation of autofluorescent compounds. Initial positives confirmed in dose response are classified into three groups based on their intracellular autofluorescence observed in the absence of the JC-1 probe: true inhibitors, autofluorescent true inhibitors, and autofluorescent compounds, such as, respectively, α-mangostin, YM 90709, and PHA 665752. **(A)** Table with chemical structure, category, calculated IC₅₀ in the JC-1 efflux assay and representative whole-well images of treated U87MG-ABCG2 cells with or without JC-1 addition are shown. Dose-response curve in the JC-1 efflux assay in the presence or absence of JC-1 probe for **(B)** α-mangostin, **(C)** YM 90709, and **(D)** PHA 665752.

resupplied positives that were not confirmed correspond to seven autofluorescent compounds such as quinacrine hydrochloride, ellipticine, acriflavinium hydrochloride, and others. These compounds yielded high green pixel intensity and increased fluorescence in the absence of the JC-1 probe (*Supplementary Table S1*; *Supplementary Data* are available online at www.liebertpub.com/adt). Among the 58 confirmed positives, 47 were characterized with IC₅₀ values for ABCG2 efflux inhibition from nanomolar to micromolar range (*Supplementary Table S2*), including the seven inhibitors of ABCG2

already known: lapatinib,²⁶ imatinib,²⁷ dipyridamole,²¹ acacetin,²⁸ apigenin,²⁹ bromocriptine mesylate,³⁰ and ivermectin^{13,31} (*Table 2*); 11 compounds that did not titrate in the dose-response study constituted static inhibitors (*Supplementary Table S3*). Among the 40 confirmed ABCG2 inhibitors inducing a dose response in our assay that were not previously characterized, we identified 16 compounds belonging to five common chemical scaffolds with previously described activity toward ABCG2 (*Table 3*). Four of them are well-described inhibitors of the ABCG2 pump: flavonoids, chromanones,

Table 3. List of Confirmed New ABCG2 Inhibitors Identified in the JC-1 Efflux Assay Belonging to Common Chemical Scaffolds

Compound name	Common chemical scaffold	HTS %I JC-1 efflux ^a	HTS %I nuclei count ^b	JC-1 efflux IC ₅₀ (μM) ^c
Dehydrorotenone	Chromone, flavonoid	102	11	0.6 ± 0.5
Dehydrodihydrorotenone	Chromone, flavonoid	72	3	1.2 ± 0.6
LY-294,002 hydrochloride	Chromone, flavonoid	75	19	2.2 ± 0.5
Irigenin trimethyl ether	Chromone, flavonoid	70	12	15 ± 1.4
Deguelin	Chromanone, flavonoid	75	14	4.0 ± 1.3
Duartin dimethyl ether	Chromanone, flavonoid	82	15	>25
Dihydrorotenone	Chromanone, flavonoid	84	19	16 ± 0.7
Deoxysappanone B 7,3'-dimethyl	Chromanone	91	9	5.2 ± 0.07
Alpha-mangostin	Chromanone	98	8	1.3 ± 0.07
PD 153035	4-anilinoquinazoline	95	−4	2.5 ± 1.5
GW 583340 dihydrochloride	4-anilinoquinazoline	103	10	4.0 ± 0.7
Prochlorperazine	Phenothiazine	79	11	>25
Fluphenazine dihydrochloride	Phenothiazine	89	4	8.0 ± 0.15
Perphenazine	Phenothiazine	91	9	>25
2-(2-amino-6-(3,4-dimethoxyphenyl)pyrimidin-4-yl)phenol	Aminopyrimidine	78	13	6.5 ± 2.9
N-(4-(4-methoxyphenyl)-6-phenylpyrimidin-2-yl)acetamide	Aminopyrimidine	78	14	5.5 ± 1.3

^aPerformance during the screen in the JC-1 efflux readout.^bPerformance during the screen in the nuclei count readout.^cCalculated IC₅₀ in the JC-1 efflux readout.

and 4-anilinoquinazolines, a common scaffold among tyrosine kinase inhibitors such as Lapatinib (Table 3).^{28,29,32,33} In addition, we identified phenothiazines known to reverse MDR³⁴ and to interact with ABCG2,³⁵ as well as aminopyrimidines, a scaffold common to the Bcr-Abl inhibitor imatinib (Tables 2 and 3). Among the 40 confirmed inhibitors, 24 were not previously described and did not belong to any scaffold with well-described activity toward ABCG2 (Table 4). Of note, among those 24 compounds were chemicals whose activity toward ABCG2 is not surprising; this is the case, for example, of vatalanib, as many tyrosine kinase inhibitors affect ABCG2 activity and/or expression,^{32,36} and avermectin B1 is closely related to ivermectin, a known ABCG2 modulator.^{13,31}

Among the most potent positives confirmed in our assay that were previously not described as ABCG2 inhibitors were dehydrodihydrorotenone (IC₅₀ = 1.2 ± 0.6 μM), a flavonoid bearing a chromone moiety with pesticide activity targeting mitochondrial complex I,³⁷ and α-mangostin, a xanthone bearing a chromanone moiety reported to suppress extracellular-signal-regulated kinase (ERK) signaling.³⁸ To confirm our findings, we tested the ability of those two com-

pounds to eliminate the side population of U87MG-ABCG2 cells as identified by FACS using the Hoechst dye. As a control, no side population was identified for compound-treated or control-treated U87MG cells, due to the inability of these cells to efficiently efflux the Hoechst dye, as expected (Fig. 7). For control-treated U87MG-ABCG2 cells, 60% of live cells were able to efflux the Hoechst dye, corresponding to the side population. In contrast, treatment with the known ABCG2 inhibitors FTC, imatinib, sunitinib, and the chemically related tyrosine kinase inhibitor lapatinib fully suppressed this side population, as expected. As an important result, the two newly identified inhibitors dehydrodihydrorotenone and α-mangostin decreased the side population to levels corresponding to U87MG control cells, confirming that they constitute potent new inhibitors of the ABCG2 transporter.

DISCUSSION

Overexpression of MDR transporters is responsible for resistance to standard chemotherapeutic treatments in refractory cancers, perhaps mediated by the existence of cancer stem cell populations such

Table 4. List of Confirmed New ABCG2 Inhibitors Identified in the JC-1 Efflux Assay Belonging to Individual Scaffolds

Compound name	Biological activity	HTS %I JC-1 efflux ^a	HTS %I nuclei count ^b	JC-1 efflux IC ₅₀ (μM) ^c
Altanserine hydrochloride	5-HT receptor antagonist	92	10	2.0±0.4
GR 127935 hydrochloride hydrate	5-HT receptor antagonist	102	5	3.0±0.8
1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole	Estrogen receptor agonist	79	–5	3.0±0.07
SB 203580 hydrochloride	p38 kinase inhibitor	76	14	6.2±0.1
Isoliquiritigenin	Natural product	75	15	3.0±2.5
Etazolate hydrochloride	Phosphodiesterase inhibitor	70	1	3.3±0.2
2-Phenylmelatonin	Melatonin receptor agonist	103	13	10±1
Aminopurvalanol A	CDK inhibitor	76	16	4.0±0.9
Fiduxosin hydrochloride	Adrenoceptor antagonist	74	–4	4.0±0.7
GW 6471	PPAR antagonist	99	19	4.5±0.7
AGK2	SIRT2 inhibitor, MRP2 inhibitor	93	19	4.5±2.8
Avermectin B1	Insecticide, Pgp inhibitor	117	1	5.5±2.5
SP600125	JNK kinase inhibitor	85	14	5.6±0.4
Vatalanib	Tyrosine kinase inhibitor	86	20	8.0±0.8
SB 242084 dihydrochloride hydrate	5-HT antagonist	130	15	9.0±1.4
CGS-15943	Adenosine receptor antagonist	83	12	18±0.9
JWH-015	Cannabinoid receptor agonist	87	21	21±3.3
Lasalocid sodium	Ionophore	127	–18	>25
Doxazosin mesylate	Adrenergic receptor antagonist	104	8	>25
NSC 625987	CDK inhibitor	94	17	>25
PSB 36	Adenosine receptor antagonist	93	20	>25
Ziprasidone hydrochloride monohydrate	Antipsychotic	88	16	>25
AMG 9810	TRPV1 receptor antagonist	84	16	>25
CPNQ	Unknown	72	1	>25

^aPerformance during the screen in the JC-1 efflux readout.^bPerformance during the screen in the nuclei count readout.^cCalculated IC₅₀ in the JC-1 efflux readout.

as in gliomas, which have been shown to overexpress ABCG2.⁴ For this reason, the identification of ABCG2 inhibitors is of great therapeutic interest, but currently available inhibitors have either failed in the clinic or are not very promising.¹⁷ The current limitations of available assays to identify inhibitors of ABC transporters among chemical libraries may be responsible for this failure, as they mostly rely on a single measurement. For flow cytometry-based assays, the readout is the efflux of a fluorescent dye, and such an assay cannot

discriminate between toxic compounds, fluorescent compounds, compounds competing with the fluorogenic substrate, and compounds affecting the expression of the transporter.¹² Another approach that is used for the identification of ABC transporters inhibitors are cytotoxicity-based assays.^{39,40} This type of assay identifies compounds that reverse cell resistance to cytotoxic drugs. Although this approach eliminates the selection of autofluorescent compounds, it will identify many cytotoxic compounds as positives.

Fig. 7. Confirmation of selected positives and control compounds in side-population fluorescence-activated cell sorting (FACS) analysis. Bar-graph quantification of the percentage of live cell side population for U87MG and U87MG-ABCG2 cells treated with compounds compared with control. The newly identified ABCG2 inhibitors α -mangostin and dehydrodihydrotene suppress the side population of cells actively effluxing Hoechst to levels similar to those observed with U87MG control cells or with U87MG-ABCG2 cells treated with control ABCG2 inhibitors FTC, imatinib, lapatinib, and sunitinib.

A recent screen relying on a bioluminescence-based readout has identified 10 known and 37 new ABCG2 inhibitors. Some of these identified inhibitors, such as gefitinib, dipyrindamole, rotenone, and flutamide, were present in our library and scored as potent hits in our assay (Supplementary Table 2). However, the low throughput and the single-parametric data collection are notable limitations of the assay.

As opposed to previously described screening approaches, we sought to develop a method that would identify inhibitors of the ABCG2 transporter among chemical libraries while discriminating toxic and autofluorescent compounds; furthermore, this method would potentially open the door to characterizing the effect of newly identified inhibitors on ABCG2 function, expression, and intracellular localization in a miniaturized format. To reach that goal, we took advantage of an HC screening approach, enabling multiplexed readouts. As a first step, our live cell assay relying on the quantification of JC-1 efflux in U87MG-ABCG2 cells measures the effect of screened compounds on the transporter efflux activity in real time, and whole-well imaging at 4 $\times$ magnification allows us to quickly capture data for the entire well, an important factor for live screens. In addition, whole-well imaging improves data accuracy and minimizes variability due to the uneven distribution of cell populations

within the well, and nuclei staining provides access to cell count for screening wells to discriminate toxic compounds.

Of note, a challenge when relying on live imaging for high-throughput screening is the reliability of imaging instruments, and due to issues with the INCA2000 light source when capturing images on day 2 for the second replicate set of data, a shift toward a higher percentage of inhibition was observed for this data set (Fig. 5C). In spite of this discrepancy, an overall good correlation was achieved between replicate sets of data ($R^2=0.74$), and we could reliably identify known and confirmed inhibitors: In a screen of 6,912 known bioactive compounds relying on our optimized efflux assay, we identified 77 unique positives that did not significantly affect cell count (Fig. 5F) and 58 compounds were confirmed among the 64 unique compounds resupplied, corresponding to a 90% confirmation rate (Supplementary Table 2); the 10% of unconfirmed positives correspond to autofluorescent compounds (Supplementary Table 1). This result demonstrates the accuracy of positive selection using our assay and the versatility of our high-content approach: We could discriminate cytotoxic and autofluorescent compounds from the same screening well (Fig. 5F). This constitutes a main advantage compared with flow cytometry-based assays that identify multiple autofluorescent compounds as hits. Among the confirmed positives, seven had been previously reported as ABCG2 inhibitors (Table 2), validating our screening assay.

Among the confirmed positives that we identified were several tyrosine kinase inhibitors (Supplementary Table 2), two of them with previously reported activity toward ABCG2: imatinib and lapatinib (Table 2).^{26,27} Interestingly, EGFR kinase inhibitors have been shown to reverse MDR by reducing the expression of ABCG2 through interference with PI3K/Akt signaling pathway,³⁶ and Akt regulates ABCG2 activity in highly tumorigenic glioma cells.²⁰ Validating our approach, we found among the confirmed positives two EGFR kinase inhibitors (lapatinib and PD 153035). In addition, the PI3K inhibitor LY 294,002 was among confirmed inhibitors, which is in agreement with published results describing that after treatment with 10 μ M LY294,002, ABCG2 rapidly translocates from the plasma membrane to the cytoplasmic compartment in freshly derived hematopoietic stem cells.⁴¹ One of the newly identified ABCG2 inhibitors, α -mangostin, is of particular interest; this compound was reported as an effective antimetastatic agent and a specific ERK1/2 inhibitor³⁸ and was confirmed as a potent ABCG2 inhibitor in our assay ($IC_{50}=1.3 \pm 0.1 \mu$ M; Fig. 6B). This result is in agreement with a study reporting the involvement of MEK/ERK signaling in the transcriptional and post-transcriptional regulation of ABCG2,⁴² and it demonstrates the ability of our assay to identify ABCG2 modulators with an alternative mechanism of action compared with functional inhibitors.

ABCG2 inhibitors can affect the efflux of the substrate through several different mechanisms or their combination: (1) interference with the transcriptional or translational regulation of ABCG2; (2) impairing transporter trafficking to the cellular membrane; (3) competition with the substrate-specific binding site; and (4) inducing post-translational degradation of the transporter. The identification of modulators that regulate the trafficking of ABCG2 and decrease its

expression level is of high interest, providing an alternative to known ABCG2 modulators that have failed in the clinic. However, most existing assays do not distinguish between the different mechanisms of action highlighted earlier. Our assay strategy addresses this issue by enabling multiplexed readouts from the same screening well, opening the door to the characterization of the effect of the newly identified inhibitors on ABCG2 expression, in addition to its function. This could be achieved by ABCG2 immunostaining and high magnification imaging, so as to distinguish those compounds that modulate ABCG2 expression and/or trafficking.

In conclusion, the results of our screen validate our live-cell HCS approach for the rapid and accurate discovery and profiling of novel ABCG2 modulators. Our assay workflow proved to be amenable to screening large chemical libraries, and our multiparametric approach enables the identification and selection of ABCG2 modulators. The next steps for our approach would be staining for ABCG2 to reveal both its expression and trafficking as a way to prioritize obtained inhibitors, and may well provide alternative drug candidates to compounds acting on ABCG2 function that have failed in the clinic.

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AUTHOR DISCLOSURE STATEMENT

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