A Fluorescent Microplate-Based Assay Workflow Enabling the Functional Characterization of Multi-Drug Resistance Transporters in Living Cells

Characterization of MDR Antiporter Expression in Tissue Culture Cell Lines

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The phenomenon of multi-drug resistance (MDR) is a well-known problem in oncology and thus needs profound consideration in any cancer treatment. An underlying mechanism for multi-drug resistance is up-regulation of the transmembrane ATP-binding cassette (ABC) transporter proteins. We describe a novel fluorescent probe that serves as a substrate for three major ABC transporter types and can serve as an indicator of MDR activity in microplate-based assays using living cells. An automated protocol to aspirate media, wash cells and dispense reagents is described which provides a convenient fluorescence microplate-based HTS format suitable for the screening of putative modulators of ABC transporters, facilitating rapid, reproducible, specific and relatively simple functional detection of MDR phenotypes.

Introduction

Multi-drug resistance (MDR) relates to resistance of tumor cells to a whole range of chemotherapy drugs with different structures and cellular targets. This phenomenon is a well-known problem in oncology and thus requires careful consideration in cancer treatment. One of the underlying molecular rationales for MDR is the up-regulation of a family of transmembrane ATP binding cassette (ABC) transporter proteins that are present in practically all living organisms [1-5]. These proteins cause chemotherapy resistance in oncology by actively extruding a wide variety of therapeutic compounds from the malignant cells. The same ABC transporters play an important protective function against toxic compounds in a variety of cells and tissues and at blood-tissue barriers.

ABC transporters also determine the general fate and effect of pharmaceutical agents inside the body. The three major types of ABC transporters are MDR1 (P-gp, P-glycoprotein, ABCB1), MRP1/2 (ABCC1/2) and BCRP/MXR (ABCG2) proteins. Various flow cytometry-based and microplate-based assays have facilitated determination of the functional expression levels of ABC transporters in living cells, but most dyes used as indicators (Rhodamine 123, DiOC2(3), calcein-AM) have limitations, since they are unable to detect all three major types of ABC transporter proteins, BCRP in particular. Dyes with broad transporter coverage (such as doxorubicin, daunorubicin and mitoxantrone) lack sensitivity due to overall dimness and thus generate a significant percentage of false negative results. We describe a novel fluorescent probe that serves as a substrate for all three ABC transporter types and can serve as an indicator of MDR in microplate-based assays using living cells. The probe exhibits fast internalization, favorable uptake/efflux kinetics and high sensitivity of MDR detection, as established by multi-drug resistance activity factor (MAF) values and Kolmogorov-Smirnov statistical analysis. Used in combination with general or specific inhibitors of ABC transporters, the workflow readily identifies functional efflux, as well as, defining the type of MDR. We have optimized the use of the EL406™ Combination Washer Dispenser to automatically aspirate media, wash cells and dispense reagents for the assay, allowing, for the first time, easy quantitation of MDR using a convenient fluorescence microplate-based HTS format that generates Z' factor values greater than 0.5. The assay can be applied to the screening of putative modulators of ABC transporters, facilitating rapid, reproducible, specific and relatively simple functional detection of MDR phenotypes.

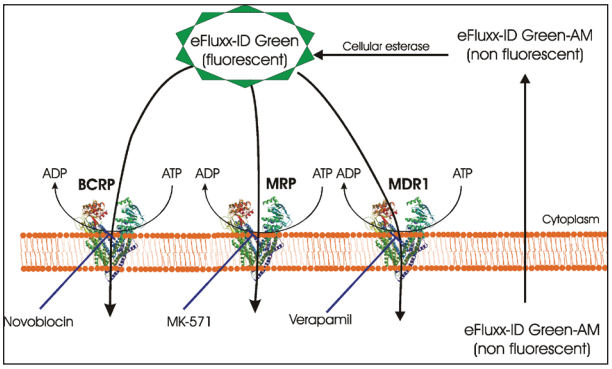

Figure 1. Schematic depiction of Multi-Drug Resistance Transporters.

The eFluxx-ID™ Green MDR Assay Kit is designed for functional detection and profiling of MDR phenotypes in live cells. The kit provides a fast, sensitive and quantitative method for monitoring the function and expression of the three clinically most important MDR proteins: MDR1 (P-glycoprotein), MRP1/2 and BCRP. The eFluxx-ID™ Green Detection Reagent, being a substrate for the three main ABC transporter proteins, serves as an indicator of these proteins' activity in the cell. The AM-ester form of the eFluxx-ID™ Green Detection Reagent is a hydrophobic non-fluorescent compound that readily penetrates the cell membrane and is subsequently hydrolyzed inside of the cells by intracellular esterases. The resulting probe is a hydrophilic fluorescent dye that is trapped within the cell unless actively pumped out by an ABC transporter (Fig 1). The fluorescence signal of the dye generated within the cells thus depends upon the activity of the ABC transporters. The cells with highly active transporters demonstrate lower fluorescence because of the active efflux of the reagent from the cell. Application of specific inhibitors of the various ABC transporter proteins, included in the kit, allows differentiation between the three common types of MDR pumps. The activity of a particular MDR transporter is defined by the difference between the amount of the dye accumulated in the presence and in the absence of the inhibitors, respectively.

Materials and Methods

CELLestial® eFluxx-ID® Green multidrug resistance assay kits were obtained from Enzo Life Sciences (Farmingdale, NY). HT1080, HepG2, and CHO-M1 cell lines were obtained from ATCC. The mesothelioma cells (LP9, H Meso, GA, and MI) were a generous gift from Nicholas Heintz at the Vermont Cancer Center and University of Vermont. For all experiments, cell lines were seeded into 96-well plates at 20,000 cells per well (100 µL) and allowed to attach overnight. The following morning the cells were treated with 40 µM verapamil (MDR1 inhibitor), 100 µM MK-571 (MRP inhibitor), 5 µM Novobiocin (BCRP inhibitor), or media (control) for 15 minutes at 37°C in a 5% CO_2 environment. Reagents were added using the EL406™ peristaltic pump such that each agent was added to 2 rows of the 96-well microplate. After drug treatment, 50 µL of eFluxx-ID® Green reagent was added using the EL406 peristaltic pump. Note that dispense cartridges were changed between the addition of drug treatment and detection reagent addition. Cells were again incubated at 37°C in a 5% CO_2 environment for 30 minutes after followed with 2 washes with 200 µL of ice-cold PBS using the EL406 washer. Finally 100 µL of PBS was added and the fluorescence was determined using a Synergy™ H4 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT). The eFluxx-ID® Green dye fluorescence was measured using an excitation of 480 nm and an emission of 530 nm. The multidrug resistance factor (MAF) was calculated using the following formula MAF = 100 X ((FE – FO)/FE) where FE is the fluorescence of the inhibitor treated samples and FO is the fluorescence of the untreated control samples.

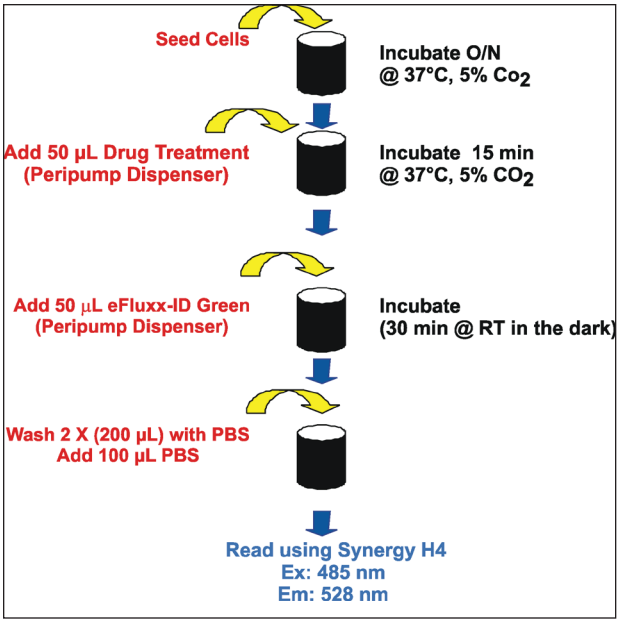

Figure 2. Schematic of the Automated eFluxx-ID® Green Assay process carried out by the EL406 Washer Dispenser (Red text) and Synergy H4 Hybrid Multi-Mode Microplate Reader (Blue text).

Results

A number of cell lines were characterized for the functional expression of three clinically important multidrug resistance proteins. All four of the well-established cell lines exhibit expression of one or more of these proteins (Fig 3). While the expression pattern for HepG2 does not exactly match that described in the literature, these cells have undergone a number of passages and may not necessarily represent the cell line described. Despite all cell lines exhibiting some form of multidrug resistance antiporter activity, the cell lines differ considerably in their activity pattern. When HepG2 cells were subjected to increasing concentrations of the MDR-1 inhibitor, verapamil, the level of fluorescence increased in a dose dependent fashion (Figure 4).

This suggests that the MDR1 is constitutively expressed at high levels; with increasing amounts of inhibitor the ability of the cell to extrude the eFluxx-ID® dye through this specific MDR protein becomes limited. As more dye is trapped within the cell the MAF value increases.

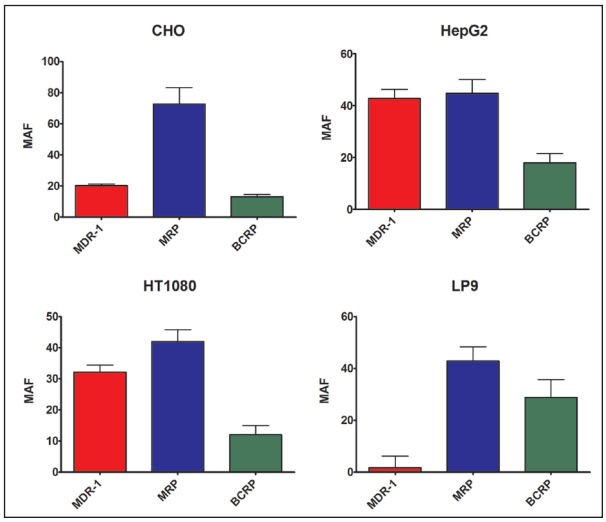

Figure 3. eFluxx-ID® Green signal from CHO-M1, HepG2, LP9 and HT 1080 cell lines.

When different mesothelial cells were profiled for the presence of multidrug resistance protein expression remarkably different patterns emerged (Figure 5). LP9 cells and H Meso cells are established cell lines with LP9 being considered to be "normal" mesothelial phenotype does not produce significant amounts of MDR-1 protein, while H Meso cells, which are considered to be a tumorigenic mesothelioma expresses all three MDR proteins tested for. MI and GA cell lines are primary cell lines recently isolated from mesothelioma tumors. Despite the commonality of the tumor the cell lines exhibit different MDR phenotypes.

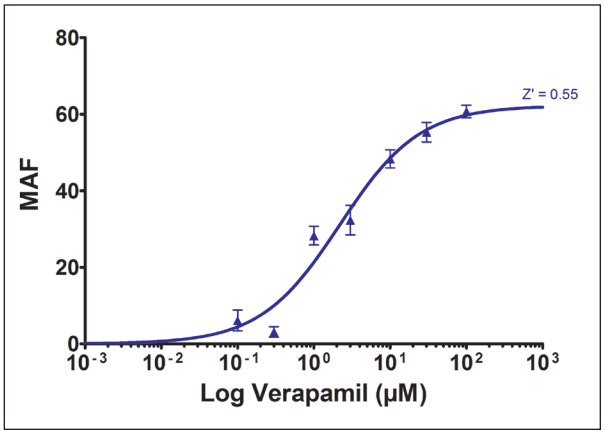

Figure 4. Effect of verapamil on MDR1 eFluxx-ID® Green signal.in HepG2 cells Note that media containing different verapamil concentrations was added using the peristaltic pump. Each concentration (up to eight) was added using a different dispense tube for an entire row.

Discussion

These data provide evidence that the eFluxx-ID® multidrug resistance assay kit from Enzo Life Sciences can be used to test cell lines for the phenotypic expression of transporter proteins. By using inhibitors specific to each of the three different antiporters, cells can be characterized as to which transporter is being expressed in terms of their multidrug resistance. The described assay is rapid, sensitive and specific. It is also compatible with standard high-throughput microplate-based screening workflows. Besides characterization of cell lines, this assay kit can be used as a means to screen compounds for the induction or inhibition of activity of multidrug resistance proteins.

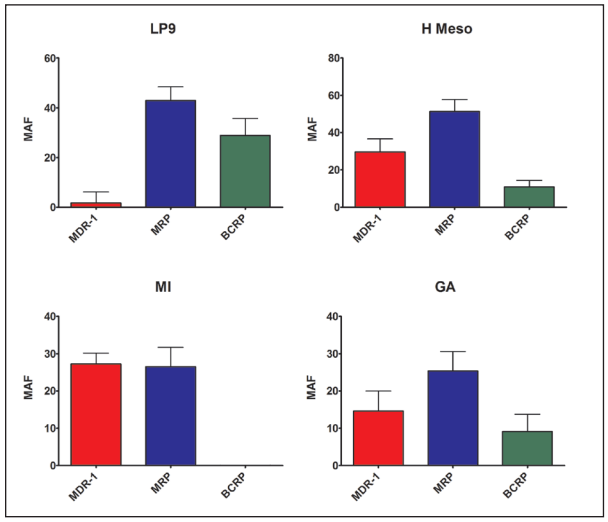

Figure 5. Characterization of different mesothelial and mesothelioma cell lines. Expression of different MDR transporters was assessed and compared using eFluxx-ID® Green dye in conjunction with specific MDR inhibitors.

We observed that different cell lines exhibited expression of one or more of the transporter proteins tested. Many of these cell lines have been in culture for decades under various physiologic and chemical stresses, which would provide a selective advantage to cells that can rapidly remove large amounts of a xenobiotic. Interestingly, cell lines recently isolated from mesothelioma tumors also demonstrate expression of the transporter proteins. These cell lines have not had the lengthy stay in culture, yet exhibit the same phenomenon. Despite being from the same tumor type the different cell lines have very different transporter phenotypes, suggesting that chemical resistance need not always follow the same path with tumor cells.

The EL406™ Washer Dispenser is capable of automating the different fluid handling steps of this and several other CELLestial® assays. The EL406 Washer Dispenser's Parallel Dispensing Technology combines several fluid handling technologies into one instrument. The patented dual-action manifold provides full plate washer functionality 96- and 384- and 1536-well microplates.

The priming trough has been replaced with a sonicator bath to provide easy cleaning of the aspiration and dispense-tubes of the washer manifold. Immediately adjacent to the full plate washer manifold is an eight channel peristaltic pump dispenser head that has eight individual tubes to provide up to 8 different fluids to a microplate. This feature was used to generate an 8-point dose response curve with verapamil. This 96- and 384-well plate capable dispenser uses autoclavable cassettes that are available in different sizes to optimize fluid dispense accuracy and precision. In addition to a low dead volume, reagents can also be recovered by reversing the direction of the peristaltic pump. Two optional syringe pump dispensers can also be utilized to dispense additional reagents.

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Acknowledgements

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