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

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Abstract

An underlying mechanism for multi-drug resistance (MDR) is up-regulation of the transmembrane ATP-binding cassette (ABC) transporter proteins. 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 fail to detect BCRP transporter protein. 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 multi-drug resistance. 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 multi-drug resistance 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.

Introduction

Multi-drug resistance relates to resistance of tumor cells to a whole range of chemotherapy drugs with different structures and cellular targets. The phenomenon of multi-drug resistance (MDR) is a well-known problem in oncology and thus needs profound 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 present in practically all living organisms[1-5]. These proteins cause chemotherapy resistance in cancer 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.

The eFluxx-ID™ Green Multidrug Resistance Assay Kit is designed for functional detection and profiling of multi-drug resistant 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 multi-drug resistance proteins: MDR1 (P-glycoprotein), MRP1/2 and BCRP. The eFluxx-ID Green Detection Reagent, being a substrate for 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. 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 will 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 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.

Multi-Drug Resistance

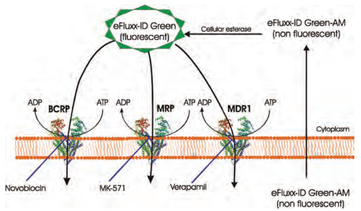

Figure 1 – Schematic depiction of Multi-Drug Resistance Transporters. The non-polar eFluxx-ID Green dye AM ester crosses the cell membrane where it is acted upon by cellular esterases. De-esterification makes the dye fluorescent as well as a substrate for three of the main transmembrane ATP binding cassette (ABC) transporter proteins (MDR1, MRP1/2, and BCRP). Specific inhibitors of these transporter proteins are also depicted.

Assay Process

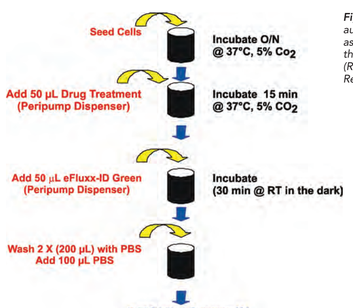

Figure 2 – Schematic of the automated eFluxx-ID Green assay process carried out by the EL406 Washer Dispenser (Red text) and Synergy™ H4 Reader (Blue text).

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 either 40 µM verapamil (MDR1 inhibitor), 100 µM MK-571 (MRP inhibitor), 5 µM Novobiocin (BCRP inhibitor), or media (control). Reagents were added using the EL406 peristaltic pump dispenser such that each agent was added to 2 rows of the 96-well microplate. Cells were incubated for 15 minutes at 37°C in a 5% CO_2 environment. After drug treatment, 50 µL of eFluxx-ID Green reagent was added using the EL406 peristaltic pump dispenser. Note that dispense cartridges were changed between the addition of drug treatment and reagent addition. Cells were again incubated at 37°C in a 5% CO_2 environment for 30 minutes after which they were washed 2 times with 200 µL of ice-cold PBS using the EL406 washer. Finally 100 µL of PBS was added and the fluorescence was then determined using a Synergy H4 reader. eFluxx-ID Green dye was measured using an excitation of 480 nm and an emission of 530 nm. The multi-drug resistance factor (MAF) was calculated.

Cell Line Characterization

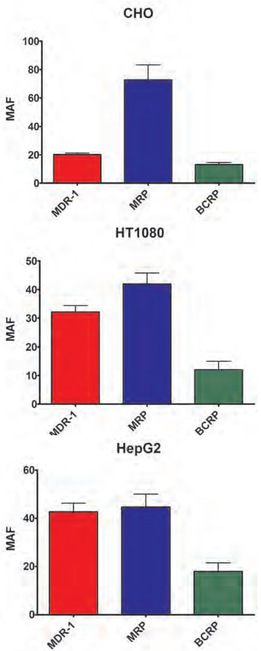

Figure 3 – eFluxx-ID Green signal from CHO-M1, HepG2 and HT 1080 cell lines. MAF = 100 X ($(F_E - F_O)/F_E$) where F_E is the fluorescence of the inhibitor treated samples and F_O is the fluorescence of the untreated control samples. In order to test for MDR-1, cells were treated with 40 µM verapamil, MRP was tested with 100 µM MK-571, and BCRP with 5 µM Novobiocin.

A number of cell lines were characterized for the functional expression of three of the clinically important multi-drug resistance proteins. All three of the well established cell lines exhibit expression of these proteins (Fig 3). While the expression pattern for HepG2 does not exactly match that described in the literature, these cell lines have undergone a number of passages and may not necessarily represent the cell line described. When cells were subjected to increasing concentrations of the MDR-1 inhibitor, verapamil the level of fluorescence increased in a dose dependent fashion (Figure 4). When different mesothelial cells were profiled for the presence of multi-drug resistance protein expression remarkably different patterns emerge (Figure 5). LP9 cells and H Meso cells are established cell lines with LP9 being considered to be "normal" mesothelial in nature does not produce significant amounts of MDR-1 protein, while H meso cells, which are considered to be a tumorigenic mesothelioma expresses all three 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.

Pharmacology

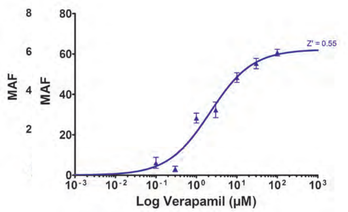

Figure 4 – Effect of verapamil on MDR1 eFluxx-ID Green signal. Note that media containing different verapamil concentrations was added using the peristaltic pump. Each concentration (up to eight) was added using a different dispense tube for an entire row. MAF = 100 X ($(F_E - F_O)/F_E$) where F_E is the fluorescence of the inhibitor treated samples and F_O is the fluorescence of the untreated control samples.

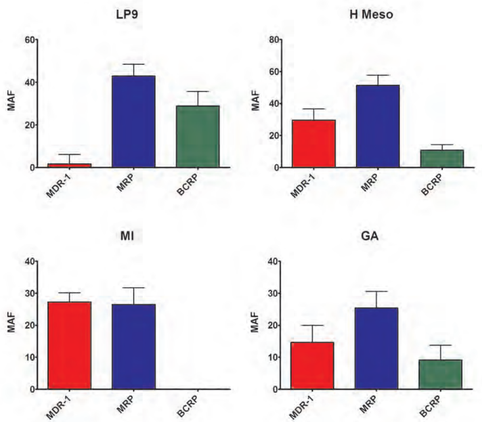

Figure 5 – Characterization of different mesothelial and mesothelioma cell lines. Expression of different MDR transporters was assessed and compared using eFluxx-ID Green dye in conjunction with specific MDR inhibitors. MAF = 100 X ($(F_E - F_O)/F_E$) where F_E is the fluorescence of the inhibitor treated samples and F_O is the fluorescence of the untreated control samples. In order to test for MDR-1, cells were treated with 40 µM verapamil, MRP was tested with 100 µM MK-571, and BCRP with 5 µM Novobiocin.

EL406 Washer Dispenser

Figure 6 – EL406 Combination Washer Dispenser.

The EL406 Combination Washer Dispenser combines several existing fluid handling technologies into one instrument. The patented dual-action manifold provides full plate washer functionality for 96-, 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 96- and 384-well plate capable dispenser uses autoclavable cassettes that come 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.

Conclusions

1. Cell lines can easily be characterized for MDR transporter protein expression.
2. Mesothelioma cells lines exhibit different phenotype patterns of multidrug resistance protein expression.
3. The CELLestial™ eFluxx-ID Kit provides an easy means to screen cells for the induction or inhibition of multidrug resistance.
4. The described assay is rapid, sensitive and specific. It is also compatible with standard high-throughput microplate-based screening workflows.
5. The EL406 Washer Dispenser is capable of automating the different fluid handling steps of several different CELLestial assays.

1. Gupta RS. (1988) Intrinsic multidrug resistance phenotype of Chinese hamster (rodent) cells in comparison to human cells. *Biochem. Biophys. Res. Commun.* 153:598-605. • 2. McCollum AK., TenEyck CJ., Stensgard B., et al. (2008) P-glycoprotein-mediated resistance to Hsp90-directed therapy is eclipsed by heat shock response. *Cancer Res.* 68:7419-7427. • 3. Hunter J., Hirst BH., Simmons NL. (1991) Epithelial secretion of vinblastine by human intestinal adenocarcinoma cell (HCT-8 and T84) layers expressing P-glycoprotein. *Br. J. Cancer* 64:437-444.
4. Hammond CL., Marchan R. , Krance SM., and Ballatoru N. (2007) Glutathione export during apoptosis requires functional multidrug resistance associated proteins. *J. Biol. Chem.* 282:14337-14347. • 5. Bodey B., Taylor CR., Siegal SE., and Kaiser HE. (1995) Immunocytochemical observation of multidrug resistance (MDR) p170 glycoprotein expression in human osteosarcoma cells. The clinical significance of MDR protein overexpression. *Anticancer Res.* 15(6B):2461-2468.