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Sensitive and Specific Probes for Discrimination of the Three Major Types of ABC Transporters

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ABSTRACT

Multidrug resistance (MDR) is well known in oncology. An underlying mechanism for MDR is up-regulation of the transmembrane ATP-binding cassette (ABC) transporter proteins, such as MDR1 (P-glycoprotein, ABCB1), MRP1/2 (ABCC1/2) and BCRP/MXR (ABCG2) proteins. ABC transporters also determine the general fate and effect of pharmaceutical agents in the body. Functional activity of these proteins may not correlate with their expression levels detected by classical methods, such as Northern blotting, RNA *in situ* hybridization, RT PCR or immunostaining. Expression levels can be below the detection threshold of some techniques, as relatively few active transporter molecules can cause major alterations in drug transport. Flow cytometry (FCM) allows determination of the actual functional expression level of MDR-ABC transporters in live cells. FCM method is based upon the uptake of any dye/drug into the cell being energy independent, but ABC transporter proteins actively extruding their substrates. Typically, live cells are loaded with the MDR probe in the presence or absence of inhibitors and then analyzed for fluorescence using FCM. Cells with increased ABC transporter activity demonstrate lower fluorescence in the absence of the specific efflux inhibitors. Increased fluorescent signal in the presence of any of the specific inhibitors indicates involvement of the corresponding efflux protein in the resistance phenomenon. Most dyes used as indicators in FCM assays (Rho123, $DiOC_2(3)$, Calcein-AM) have limited applicability as they do not detect the three major types of ABC transporters at once. Dyes with broad specificity (such as doxorubicin, daunorubicin and mitoxantrone) lack sensitivity due to overall dimness and may yield a significant percentage of false negative results. We developed two fluorescent probes that are substrates for all three common types of ABC transporters and can serve as indicators of MDR in FCM assays using live cells. The probes demonstrate fast internalization, favorable uptake/efflux kinetics and high sensitivity of MDR detection as established by MAF and Kolmogorov-Smirnov statistical analysis. Used in combination with general or specific inhibitors of ABC transporters, both dyes readily identify functional efflux and can detect small degrees of efflux as well as define the type of multidrug resistance. The assay should be applicable to the screening of putative modulators of MDR-ABC transporters. The probes' spectral characteristics are compatible with those of dyes commonly in use with multicolor FCM. The described assay workflow facilitates rapid, reproducible, specific and relatively simple functional detection of ABC transporter activity, high-volume specimen throughput and can readily be implemented on widely available instrumentation.

INTRODUCTION

Multidrug resistance (MDR) relates to resistance of tumor cells to a whole range of chemotherapy drugs with different structures and cellular targets. MDR presents a well known problem in oncology that warrants profound consideration in planning various cancer treatments. One of the underlying molecular rationales for MDR is the up-regulation of a family of MDR transporter proteins that cause chemotherapy resistance in cancer by actively extruding a large variety of therapeutic compounds from the malignant cells. MDR transporters belong to the evolutionarily conserved family of the ATP binding cassette (ABC) proteins, presented in practically all living organisms from prokaryotes to mammals. The same ABC transporters play an important protective function against toxic compounds in a variety of cells and tissues, especially in secretory organs, at the sites of absorption, and at blood-tissue barriers. The three major multidrug resistance ABC proteins are MDR1 (P-glycoprotein, ABCB1), multidrug resistance associated protein 1 (MRP1, ABCC1) and ABCG2 (placenta-specific ABC transporter, ABCP/breast cancer resistance protein, BCRP/mitoxantrone resistance protein, MXR). MDR1 and MRP1 can transport a large variety of hydrophobic drugs, and MRP1 can also extrude anionic drugs or drug conjugates. The transport properties of ABCG2 are overlapping both with that of MDR1 and the MRP type proteins, thus these three proteins form a special network in chemo-defense mechanisms .

Because of a significant role that ABC transporters play in cancer multidrug resistance and the body's protection against xenobiotics, sensitive and specific quantitative assays are required for the detection of the activity of these proteins. Also, high throughput assay systems are required to screen for potential transporter-interacting partners. The estimation of the activity of ABC transporter is not easily achieved by the routinely available classical non-functional methods, such as Northern blotting, RNase protection, RNA *in situ* hybridization, RT PCR or immunostaining. MDR-ABC protein expression is often not correlated with mRNA levels or is often below the detection threshold, as relatively few active transporter molecules may cause major alterations in drug transport. Additionally, functional activity of ABC transporters may not correlate with their expression levels determined by the methods listed above.

CLINICAL SIGNIFICANCE

Clinicians are especially interested in identifying the drug resistance profile, the substrate specificity and drug extrusion activity of the various multi-drug resistance proteins expressed in a given tumor sample. The demonstration of the transport activity of various multi-drug resistance proteins in the plasma membrane requires a sensitive and reproducible *in vitro* multi-drug resistance assay. To date, BCRP has gained prominence as one of the three major ATP-binding cassette (ABC) membrane efflux transporters alongside MDR1 and MRP, conferring drug resistance in cancer and inflammation chemotherapies. Besides being present in drug-resistant cancer and T-cells, BCRP is also endogenously expressed at a high level in human placenta and to a lesser extent in liver, small intestine and colon, ovary, veins, capillaries, kidney, adrenal, and lung, with little to no expression in brain, heart, stomach, prostate, spleen, and cervix. It has been demonstrated that calcein AM is useful for the quantitative functional analysis of the presence of active MDR1 and MRP but not BCRP transporters in cells. Thus, it is important to have a simple, reproducible and sensitive method to determine activity of all three types of ABC transporters in live cells.

There is need for a general non-toxic MDR probe that:

1. Recognizes all three major ABC transporter's types in live cells (unlike the most common MDR probe Calcein AM)
2. Is more sensitive than common general MDR probes, such as doxorubicin and mitoxantrone
3. Requires few processing steps and provides a reproducible protocol

FIGURE 1: A key physiological function of ABC transporters is the protection of cells from many toxic insults from either endogenous or exogenous molecules that can enter the cell by diffusion or active uptake . The protective mechanism afforded by ABC transporter mediated extrusion of such toxic substances, whether they are metabolic waste products, naturally occurring substances or drugs, can make tumor cells resistant to the toxic effects of various chemotherapeutic agents (Fletcher, Nature Rev Cancer, 2010)

ABC Transporter	Gene subfamily	Substrates of the protein	Reversal/Agents*	Probes for Detection*
MDR1	MDR/TAP/CBF	Anthracyclines (DOX, DNR), colchicines, vinca alkaloids, etoposide, taxanes	Verapamil, CsA, reserpine, Cyclosporin A, PSC833[6]	$DiOC_2(3)$, Rho 123, DOX, Calcein-AM, JC-1
MRP	MRP/ABCC	Anthracyclines (DOX, DNR), camptothecin, vinca alkaloids, methotrexate, etoposide, **N O T T A X A N E S**	Cyclosporin A, probenecid, indomethacin MK-571, CsA	Fluo-3, DOX, DNR, Calcein-AM, SNARF,
BCRP	ABCG	Anthracyclines (DOX, DNR), mitoxantrone, topotecan, [prosystin], methotrexate, **N O T v i n c a A lk a l o i d s , t a x a n e s**	Fumitremorgin C (specific BCRP inhibitor), GF120918, Cyclosporin, CsA, reserpine, tamoxifen, [prosystin]	Rho 123, JC-1, Mxt, DNR, BODIPY®-prazosin, bisantrene, Hoechst 33342, LysotrackerGreen

TABLE 1: Substrate and inhibitor specificity of the major ABC transporters

FIGURE 3: Venn diagram of the fluorescent substrates previously used for studying MDR-ABC transporters function. Some of these substrates are selective for one or two transporters, others have overlapping specificities.

METHODS

- Tested cells were trypsinized, washed twice in PBS and aliquotted at a density of $1x10^6$ cells/ml. All validated cell lines are listed in Table 2
- Dye uptake protocol was used :
  - Incubation of the cells with or without pump inhibitors for 5 min, 37°C
  - Loading the cells with the indicated probe for 30 min, 37°C, with or without pump inhibitors.
  - Optional steps:
    - ✓ Collect cells by brief centrifugation and re-suspend them in ice-cold PBS
    - ✓ Wash and re-suspend with ice-cold PBS
    - ✓ Keep on ice until the assay
  - Analyze using flow cytometry
- Inhibitors of MDR used for assay development
  - Verapamil (specific Pgp inhibitor) – 20 μM
  - Cyclosporin A (general MDR inhibitor) – 5 μM
  - Probenecid (specific MRP inhibitor) - 0.2 mM
  - MK-571 (specific MRP inhibitor) - 0.05 mM
  - Novobiocin (specific BCRP inhibitor) – 0.05 mM
  - Fumitremorgin C (specific BCRP inhibitor)-10 μM
- After the incubation, cells were analyzed by flow cytometry. Mean values of fluorescence were registered for each sample.
- MAF values were calculated for each sample. Additionally, histograms were analyzed using Kolmogorov-Smirnov statistics (see below). The sample was considered to be positive if the D value >0.2

KOLMOGOROV-SMIRNOV & MAF STATISTICS

- Kolmogorov-Smirnov statistics [13] measures the difference between two distribution functions and generates a D value ranging between 0 and 1.0, with higher values indicating a greater difference between the distribution functions.
- The D value generated by the KS statistics was modified by ascribing a negative value if the fluorescence of a negative control (no inhibitor) was brighter than the fluorescence of the sample with inhibitor.
- The method was one of those recommended by the First International MDR1 Methods Detection Workshop [5].
- KS values appear to be a reliable method for accurately measuring of MDR. Intensity staining was categorized as follows (for $DiOC_2$ and MRP detection):
  - Bright ($D > 0.30$)
  - Moderate ($0.20 < D < 0.30$)
  - Dim ($0.15 < D < 0.20$)
  - Negative ($D < 0.15$)
  - Notably, D-values cut-points may vary slightly (about 10%) based upon staining different proteins with different dyes.
- In the following tables, D-value for each protocol and each inhibitor were compared to MAF values (MDR Activity Factor) that were calculated using the following formula: $MAF=100*(MFI_{inh}-MFI_{neg})/MFI_{inh}$), where MFI_{inh} and MFI_{n} are mean fluorescence intensity values measured in the presence and absence of inhibitor. The cut-off values of MAF for Calcein AM adopted in clinical assays are about 20-25.

Cell Line (Reference)	ABC Transporters		
	MDR1	MRP	BCRP
CHO K1 [6]	+	+	+
HeLa [6]	-	-	-
A549 [7]	-	+	+
HCT-8 [8, 9]	+	+	-
Hep-G2 [10]	+	-	-
Jurkat [11]	-	+	-
U-2 OS [12]	+	+	+
U-2 OS GFP	+	+	+
U-2 OS RFP	+	+	+

TABLE 2: The eFluxx-ID™ Multidrug resistance Assay Kit has been validated in various cell lines expressing multidrug resistance proteins

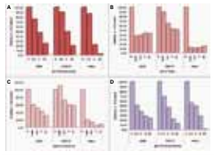

FIGURE 3A-D: CHO K1 and A549 cell lines display increased chemoresistance toward the cytotoxic drugs that are mostly associated with MDR: doxorubicin (panel A), Taxol (panel B), Vincristine (panel C), mitoxantrone (panel D). HeLa cell line was used as a non-chemoresistant control. Cells were seeded in 96 well plates, treated with the different doses of indicated drugs, and standard MTT test was performed 1 and 2 days post-treatment. Results are presented as a ratio of the OD_{595} of treated cells to the OD_{595} of the untreated cells.

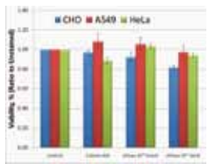

FIGURE 4: eFluxx-ID™ Green and Gold detection reagents used for the MDR assay are not toxic to the cells. CHO K1, A549 and HeLa cells were seeded in 96-well plates and next day treated with different fluorescent dyes (Calcein AM was used as a control) at working concentrations (see Materials and Methods) for 3 hours at 37°C. Then standard MTT test was performed and cellular viability was calculated as a ratio of OD_{595} of treated cells to OD_{595} of untreated cells

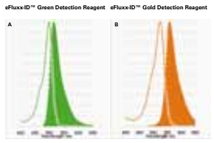

FIGURE 5: Spectral characteristics of the eFluxx-ID™ Green, EX/EM 490/514 (A) and Gold, EX/EM 530/570 (B) reagents.

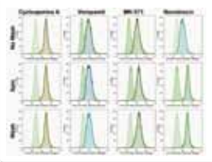

FIGURE 6: Optimization of the dye uptake assay using eFluxx-ID™ Green Detection Reagent and CHO K1 cells. Washing steps are optional and does not affect assay results.

Value	Protocol	Cyclosporin A	Verapamil	Probenecid	Novobiocin
MAF	No Wash	93.0	77.6	85.4	93.5
	Spin	91.5	73.0	85.0	92.4
	Wash	91.6	73.5	86.2	91.3
D-values	*No Wash*	*0.97*	*0.81*	*0.9*	*0.98*
	Spin	*0.94*	*0.76*	*0.9*	*0.97*
	Wash	*0.94*	*0.76*	*0.87*	*0.94*

TABLE 3: Optimization of the dye uptake protocol for the MDR assay, CHO K1 cell line, eFluxx-ID™ Green Detection reagent.

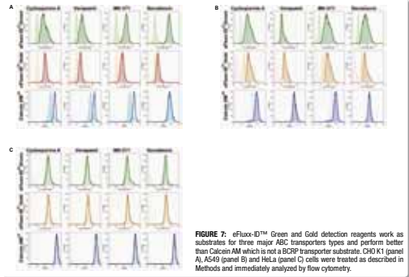

FIGURE 7: eFluxx-ID™ Green and Gold detection reagents work as substrates for three major ABC transporters types and perform better than Calcein AM which is not a BCRP transporter substrate. CHO K1 (panel A), A549 (panel B) and HeLa (panel C) cells were treated as described in Methods and immediately analyzed by flow cytometry.

Cell Line	Probe	Cyclosporine A	Verapamil	Probenecid	Novobiocin
CHO K1	Calcein AM	0.96	0.93	0.70	0.06
	e-Fluxx-ID™ Green	0.96	0.83	0.89	0.78
	e-Fluxx-ID™ Gold	0.96	0.89	0.74	0.80
A549	Calcein AM	0.47	0.15	0.27	0.03
	e-Fluxx-ID™ Green	0.51	0.09	0.49	0.44
	e-Fluxx-ID™ Gold	0.42	0.12	0.28	0.40
HeLa	Calcein AM	0.14	0.13	0.09	0.09
	e-Fluxx-ID™ Green	0.14	0.04	0.15	0.09
	e-Fluxx-ID™ Gold	0.05	0.04	0.03	0.06

TABLE 4: Detection of MDR activity by flow cytometry method (dye uptake) in model cell lines. Activity of ABC transporters is expressed as D-values (Kolmogorov-Smirnov statistics).

Cell Line	MDR Probe	Specificity	Verapamil	Probenecid	FTC
CHO K1	Calcein AM	MDR1, MRP	0.74	0.77	0.17
	eFluxx-ID Green	MDR1, MRP, BCRP	0.84	0.69	0.48
	eFluxx-ID Gold	MDR1, MRP, BCRP	0.80	0.58	0.32
	$DiOC_2(3)$	MDR1	0.96	0.01	0
	Phesphorbide A	BCRP	0.02	0.03	0.25
	CMFDA	MRP	0.19	0.7	0.09
A549	Calcein AM	MDR1, MRP	0.17	0.36	0.04
	eFluxx-ID Green	MDR1, MRP, BCRP	0.07	0.26	0.23
	eFluxx-ID Gold	MDR1, MRP, BCRP	0.14	0.21	0.29
	$DiOC_2(3)$	MDR1	0.07	0	0.03
	Phesphorbide A	BCRP	0.16	0.16	0.35
	CMFDA	MRP	0.04	0.29	0.21
HeLa	Calcein AM	MDR1, MRP	0.17	0.06	0.03
	eFluxx-ID Green	MDR1, MRP, BCRP	0.10	0	0.15
	eFluxx-ID Gold	MDR1, MRP, BCRP	0	0	0.10
	$DiOC_2(3)$	MDR1	0	0.06	0.07
	Phesphorbide A	BCRP	0	0	0
	CMFDA	MRP	0	0.03	0.12

TABLE 5: Comparison of MDR activity detection in model cell lines using MDR probes and inhibitors of different specificity. Average D-values for three representative experiments are provided.

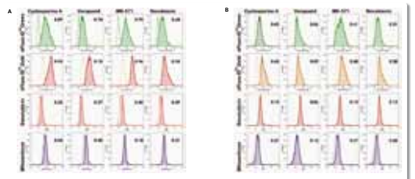

FIGURE 8: eFluxx-ID™ Green and Gold detection reagents recognize three major types of ABC transporters similarly to Doxorubicin and Mitoxantrone probes but demonstrated much higher sensitivity then latters. Model cell lines (CHO K1, panel A, and A549, panel B) were treated as described in Methods and immediately analyzed by flow cytometry. Population comparison was performed using Kolmogorov-Smirnov statistics [13]

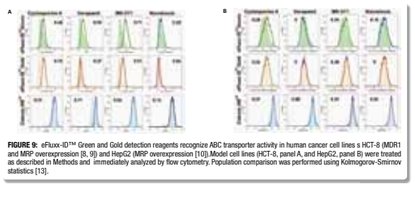

FIGURE 9: eFluxx-ID™ Green and Gold detection reagents recognize ABC transporter activity in human cancer cell lines s HCT-8 (MDR1 and MRP overexpression [8, 9]) and HepG2 (MRP overexpression [10]).Model cell lines (HCT-8, panel A, and HepG2, panel B) were treated as described in Methods and immediately analyzed by flow cytometry. Population comparison was performed using Kolmogorov-Smirnov statistics [13].

CONCLUSIONS

- We describe a sensitive and quantitative method for monitoring multidrug resistance in live cells
- The eFluxx-ID™ MDR dyes detect and distinguish between three major clinically important multidrug resistance proteins: MDR1 (P-glycoprotein), MRP and BCRP
- The assay employs a simple and reproducible mix-and-read protocol, optimized for flow cytometry
- The assay allows quantification and comparison of multidrug resistance levels between different samples or cell lines.
- The 488 nm laser excitable eFluxx-ID™ dyes are compatible with a wide range of instruments, and with other common fluorescent dyes/fluorescent proteins commonly used in flow cytometry
- Fluorescent signal does not depend upon changes in the intracellular milieu (i.e. pH and Ca^{2+} concentrations)
- The assay should be applicable to multidrug resistance analysis and drug/toxicity investigations.

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