LysoTracker and MitoTracker Red are transport substrates of P-glycoprotein: implications for anticancer drug design evading multidrug resistance

Benny Zhitomirsky #, Hodaya Farber #, Yehuda G. Assaraf *

Department of Biology, The Fred Wyszkowski Cancer Research Laboratory, Technion-Israel Institute of Technology, Haifa, Israel

Received: September 5, 2017; Accepted: November 2, 2017

Abstract

LysoTracker and MitoTracker Red are fluorescent probes widely used for viable cell staining of lysosomes and mitochondria, respectively. They are utilized to study organelle localization and their resident proteins, assess organelle functionality and quantification of organelle numbers. The ATP-driven efflux transporter P-glycoprotein (P-gp) is expressed in normal and malignant tissues and extrudes structurally distinct endogenous and exogenous cytotoxic compounds. Thus, once aromatic hydrophobic compounds such as the above-mentioned fluorescent probes are recognized as transport substrates, efflux pumps including P-gp may abolish their ability to reach their cellular target organelles. Herein, we show that LysoTracker and MitoTracker Red are expelled from P-gp-overexpressing cancer cells, thus hindering their ability to fluorescently mark target organelles. We further demonstrate that tariquidar, a potent P-gp transport inhibitor, restores LysoTracker and MitoTracker Red cell entry. We conclude that LysoTracker and MitoTracker Red are P-gp transport substrates, and therefore, P-gp expression must be taken into consideration prior to cellular applications using these probes. Importantly, as MitoTracker was a superior P-gp substrate than LysoTracker Red, we discuss the implications for the future design of chemotherapeutics evading cancer multidrug resistance. Furthermore, restoration of MitoTracker Red fluorescence in P-gp-overexpressing cells may facilitate the identification of potent P-gp transport inhibitors (i.e. chemosensitizers).

Keywords: P-glycoprotein • LysoTracker • MitoTracker • cancer • drug-resistance

Introduction

Fluorescence microscopy plays a major role in the field of cell biology and physiology. A wide array of viable molecular markers that target specific organelles and emit fluorescence are an integral part of the toolbox of modern biomedical research. However, various fluorescent dyes were previously shown to be expelled from cells via ATP-driven efflux pumps, thus preventing them from reaching their target organelles and limiting their application [1–3].

LysoTracker Red DND-99 is a hydrophobic weak base and a viable fluorescent marker, which selectively accumulates in acidic vesicular compartments, predominantly in late endosomes and lysosomes [4]. LysoTracker Red DND-99 is a hydrophobic weak base and a viable fluorescent marker, which selectively accumulates in acidic vesicular compartments, predominantly in late endosomes and lysosomes [4].

LysoTracker Red contains several aromatic rings with a positively charged nitrogen at acidic pH [Fig. 1A]. Due to its hydrophobic nature (Log P = 2.1 [5]), LysoTracker Red enters cells via simple diffusion. Remarkably, owing to the acidic pH of the lysosome, upon entry into the lysosome, LysoTracker Red becomes protonated and hence is markedly sequestered in lysosomes. Applications of LysoTracker Red include, for example, colocalization of fluorescent drugs or fluorescently marked proteins with lysosomes to demonstrate their lysosomal localization [6, 7], tracking the localization of lysosomes within the cell [8] and quantification of the number of lysosomes in cells using fluorescence microscopy and computational analysis [9]. Moreover, due to the fact that lysosomal acidity plays a crucial role in the accumulation of LysoTracker inside this acidic organelle, its fluorescence intensity may serve as an indication of alterations in lysosomal pH [10]. Furthermore, a recent study suggested that LysoTracker dyes can be used for flow cytometric studies of autophagy, demonstrating that LysoTracker Green signal is enhanced during autophagic process in a...
manner comparable to the bona fide autophagy marker LC3B in Jurkat T-cell leukaemia and K562 erythromyeloid leukaemia cells [11, 12].

MitoTracker Red CMXRos is a red fluorescent dye which accumulates in mitochondria in viable cells [13] and has an excitation wavelength of 578 nm and emission of 599 nm. The hydrophobic (Log $P = 1.15$ [14]) cationic properties of MitoTracker Red allow it to selectively accumulate in mitochondria due to the negative mitochondrial membrane potential. MitoTracker Red has a large multi-aromatic structure with two positively charged nitrogens (Fig. 1B). MitoTracker Red has a wide spectrum of applications including the study of the morphology and functional state of mitochondria [15], quantification of the number of mitochondria per cell [16], colocalization of proteins within the organelle [17] and detection of apoptotic cells using flow cytometric analysis via decreased fluorescence of MitoTracker Red due to mitochondrial disintegration [18].

The ATP-binding cassette (ABC) is a superfamily of ATP-driven efflux transporters, containing multiple transmembrane proteins that actively translocate multiple substrates across biological membranes [19, 20]. ABC transporters play an important physiological role in protecting cells from numerous endogenous and exogenous toxic compounds and as such have been found to contribute to cancer multidrug resistance (MDR) by preventing various chemotherapeutic agents from entering cancer cells and exerting their cytotoxic activity [18, 21]. The first member of the ABC superfamily to be identified was P-glycoprotein (P-gp) [22–24], the product of the MDR1 gene. P-gp displays a poly-specific substrate recognition pattern hence extruding a wide spectrum of structurally and mechanistically distinct molecules in a wide range of molecular weights ranging from 330 to 4000 Da [25–27]. The majority of P-gp substrates are hydrophobic and thus markedly accumulate in the plasma membrane. It is well-established that P-gp acts as a hydrophobic ‘vacuum cleaner’ pump which binds hydrophobic substrates dissolved in the plasma membrane hence extrude them out of the cell, thus abolishing their entry into the cytoplasm and their arrival at their cellular target sites [28–30]. P-gp has two transmembrane domains containing 12 transmembrane helices, which serve as the substrate binding sites [25]. P-gp forms a natural barrier against toxins and xenobiotics in various human organs, but slight alterations such as polymorphisms in exons of the MDR1 gene may endow this pump with the ability to recognize different anticancer drugs [31], thus, rendering tumour cells MDR to chemotherapy [32, 33]. In addition to various cytotoxins and chemotherapeutic agents, ABC transporters including P-gp were also shown to expel various molecular markers, such as the viable DNA dye Hoechst 33342, the oxidative stress indicator CellROX, the mitochondrial marker MitoTracker Green and the mitochondrial membrane potential probe JC-1 [2, 34]. This extrusion of fluorescent molecular markers hinders their application in cells expressing these MDR pumps resulting in misinterpretation of the findings if this extrusion is not taken into account.

Here, we employed human P-gp-overexpressing cancer cell lines to demonstrate that the two widely used fluorescent probes LysoTracker Red and MitoTracker Red are transport substrates of P-gp, hence hindering their ability to fluorescently mark lysosomes and mitochondria in P-gp-overexpressing cells, respectively. We further demonstrate that these molecular stains are not substrates of two other MDR pumps—breast cancer resistance protein (BCRP) and multidrug resistance protein-1 (MRP1).

Materials and methods

Chemicals

LysoTracker Red DND99 and MitoTracker Red CMXRos were purchased from Invitrogen (Carlsbad, CA, USA). Hoechst 33342 was obtained from Molecular Probes (Eugene, OR, USA). Taridiguidar was purchased from MedKoo Bioscience (Chapel Hill, NC, USA). K0143 was purchased from Sigma-Aldrich (St. Louis, MO, USA), and MK571 was purchased from Cayman Chemicals (Ann Arbor, MI, USA).

Cell culture

The cell lines used in this study were as follows: SW-1573 human lung carcinoma cells and their doxorubicin-resistant subline SW-1573/2R160 [35]; EPG85-257P human gastric carcinoma cells and their daunorubicin-resistant subline EPG85-257-RDB [36]; 2008 human ovarian
carcinoma cells and their subline 2008/MRP1 stably infected to overexpress the ABCG1 gene [37]; A549 non-small cell lung cancer cells and their MDR BCRP-overexpressing subline A549/K1.5 [38]. All cell lines used in this study were maintained in RPMI-1640 medium (Gibco, Paisley, UK), supplemented with 10% foetal bovine serum, 2 mM glutamine, 100 μg/ml penicillin and streptomycin (Biological Industries, Beit HaEmek, Israel) in a humid atmosphere containing 5% CO2 at 37°C.

Membrane protein extraction and Western blot analysis

Membrane proteins were extracted from the different tumour cell lines as previously described [39]. Protein content was determined using the Bio-Rad protein assay. P-gp, MRPI and BCRP protein levels, as normalized to Na+/K+-ATPase, were determined by Western blot using JSB-1 anti-P-gp, MRPI anti-MRPI and BXP-53 anti BCRP monoclonal antibodies (kindly provided by Prof. R. J. Schepers and Dr. G. L. Scheffer, VU University Medical Center, Amsterdam, The Netherlands) and the affinity purified polyclonal anti-Na+/K+-ATPase antibody (KETTY, kindly provided by Prof. S. J. D. Karlish) as previously described [39]. The membrane was then reacted with horseradish-peroxidase conjugated secondary antibodies (Jackson Immunoresearch Labs, West Grove, PA, USA) and enhanced chemiluminescence (ECL) detection was performed according to the manufacturer’s instructions (Biological Industries).

Live cell microscopy

For live cell imaging, tumour cells were plated in 24-well glass bottom plates (In Vitro Scientific, CA, USA) at a concentration of 10⁵ cells/well in 1 ml growth medium and allowed to attach overnight. Cells were incubated for 1 hr with 100 nM LysoTracker Red or 100 nM MitoTracker Red at 37°C, in the presence or absence of the appropriate ABC transporter inhibitors; 100 nM Tariquidar for P-gp-expressing cells, 20 μM MK571 for MRPI-expressing cells and 700 nM Ko143 for BCRP-expressing cells. To achieve nuclear DNA staining prior to fluorescence imaging, cells were incubated with 2 μg/ml Hoechst 33,342 in growth medium for 10 min. Subcellular fluorescence was detected using the InCell analyser 2000 (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) fluorescence microscope.

Computational and statistical analysis

Computational analysis of LysoTracker Red and MitoTracker Red fluorescence intensity was determined using the InCell investigator software, analysing 20 fields from each well, containing at least 200 cells. The statistical analysis of computational results was performed with one-tailed-coupled Student’s t-test. P-value lower than 0.05 was considered statistically significant.

Results

To determine whether or not LysoTracker and MitoTracker Red are transport substrates of P-gp, SW-1573 human lung carcinoma cells and their doxorubicin-resistant P-gp-expressing subline SW-1573/2R160 [35], as well as EPG85-257P human gastric carcinoma cells and their daunorubicin-resistant P-gp-overexpressing subline EPG85-257-RDB [36] were used. P-gp levels in these paired tumour cell lines were determined by Western blot analysis (Fig. 2). Neither of the parental tumour cell lines expressed any detectable levels of P-gp, whereas SW-1573/2R160 cells displayed a relatively low expression level of P-gp which appeared as a ~170 kD protein; in contrast, EPG85-257-RDB expressed very high levels of P-gp (Fig. 2).

To determine whether or not LysoTracker and MitoTracker Red are transport substrates of P-gp, these two pairs of cognate tumour cell lines were incubated with 100 nM of each of the two fluorescent probes for 1 hr in the presence or absence of the potent P-gp transport inhibitor tariquidar (100 nM) [40]. Lysosomes in parental EPG85-257P cells were successfully stained with LysoTracker Red (Fig. 3, Panels A and I) and tariquidar had no significant effect on lysosome staining in these P-gp-lacking cells (Fig. 3, Panels B and I). In contrast, LysoTracker Red failed to stain lysosomes in P-gp-overexpressing EPG85-257-RDB cells, and no detectable red fluorescence was found in these cells (Fig. 3 Panels C and I). Co-incubation of EPG85-257-RDB cells with LysoTracker Red and tariquidar partially restored the lysosomal LysoTracker staining (Fig. 3 Panels D and I) when compared to the fluorescence intensity in parental EPG85-257P cells. These results indicate that LysoTracker Red is a substrate of P-gp, and hence, cells expressing high levels of this efflux pump will not be stained by LysoTracker without inhibition of P-gp efflux activity. The partial restoration of LysoTracker staining in EPG85-257-RDB cells incubated with tariquidar might be due to partial inhibition of P-gp or due to actual lower lysosome content in these cells compared to their parental cell line. Surprisingly, no such effect was found in P-gp-expressing SW-1573/2R160 cells, as the level of LysoTracker fluorescence in these cells was similar to the level of LysoTracker staining in parental SW-1573 cells and was not significantly altered by addition of

![Image](http://example.com/image.png)
tariquidar (Fig. 3 Panels E-H, and Panel I). This result suggests that the moderate P-gp levels in SW-1573/2R160 cells were not sufficient to efficiently extrude LysoTracker Red from these MDR cells.

MitoTracker Red successfully stained mitochondria in parental EPG85-257P cells (Fig. 4, Panels A and I), and this fluorescence staining was not affected by tariquidar (Fig. 4, Panels B and I).

In contrast, MitoTracker Red failed to stain P-gp-overexpressing EPG85-257-RDB cells in which no MitoTracker fluorescence was detected (Fig. 4, Panels C and I). Similar to LysoTracker Red staining, MitoTracker Red staining in EPG85-257-RDB cells was partially restored in cells co-incubated with tariquidar, indicating that MitoTracker Red is also a transport substrate of P-gp (Fig. 4 Panels D and H).

**Fig. 3** LysoTracker Red is extruded from cells via P-gp. EPG85-257P, EPG85-257-RDB, SW-1573 and SW-1573/2R160 cells were incubated with LysoTracker Red (100 nM) for 1 hr, in the presence or absence of tariquidar (100 nM). Hoechst 33342 (2 μg/ml) was used to stain nuclei. Cellular fluorescence was followed by an InCell Analyzer fluorescence microscope (A–H). LysoTracker Red fluorescence intensity was quantified using InCell investigator software. Error bars indicate standard deviation (I). *Student’s t-test P-value < 0.05 **P-value < 0.01.
The partial restoration of MitoTracker Red staining by tariquidar in EPG85-257-RDB cells, combined with the partial restoration of LysoTracker Red staining, suggests that tariquidar only partially inhibited P-gp transport activity in these cells. Unlike with LysoTracker Red, MitoTracker Red staining was significantly reduced in SW-1573/2R160 cells when compared to parental SW-1573 cells and was

**Fig. 4** MitoTracker Red is secreted from cells via P-gp. EPG85-257P, EPG85-257-RDB, SW-1573 and SW-1573/2R160 cells were incubated with MitoTracker Red (100 nM) for 1 hr, in the presence or absence of tariquidar (100 nM). Hoechst 33342 (2 μg/ml) was used to stain nuclei. Cellular fluorescence was studied using an InCell Analyzer fluorescence microscope (A–H). MitoTracker Red fluorescence intensity was quantified using InCell investigator software. Error bars indicate standard deviation (I). *Student’s t-test P-value < 0.05 **P-value < 0.01.
successfully restored by co-incubation with tariquidar (Fig. 4, Panels E-H and I), suggesting that MitoTracker Red is a much better P-gp substrate than LysoTracker Red, as even the low levels of P-gp in SW-1573/2R160 cells were sufficient to significantly reduce MitoTracker accumulation in these cells.

We have further determined whether or not LysoTracker Red and MitoTracker Red are substrates of other major efflux pumps of the ABC superfamily including MRPI and BCRP. To this end, we employed 2008/MRP1 human ovarian carcinoma cells stably infected to overexpress the ABCG1 gene which encodes for the MDR efflux transporter MRPI [37] and the MDR non-small-cell lung cancer cells A549/K1.5 which overexpress the MDR pump BCRP encoded by the ABCG2 gene [38]. The protein levels of MRPI and BCRP were determined in these cell lines using Western blot analysis (Fig. 5 Panels A, B); this confirmed that 2008/MRP1 and A549/K1.5 cells overexpress MRPI and BCRP, respectively. Fluorescence staining experiments with LysoTracker Red and MitoTracker Red were performed as described above. MKS71 (20 \mu M) was used as an MRPI transport inhibitor in 2008/MRP1 cells, whereas K0143 (700 nM) was used as a BCRP transport inhibitor in A459/K1.5 cells (Fig. 6). Both LysoTracker Red and MitoTracker Red successfully stained lysosomes and mitochondria, respectively, in both parental and MDR tumour cell lines. Inhibition of MRPI in 2008/MRP1 cells as well as inhibition of BCRP in A549/K1.5 cells did not alter LysoTracker and MitoTracker Red staining. These results demonstrate that these fluorescent probes are neither substrates of MRPI nor of BCRP.

**Discussion**

Our present findings establish that the viable intracellular fluorescent markers LysoTracker Red and MitoTracker Red are transport substrates of P-gp. From a physiological perspective, P-gp is expressed at substantial levels in different tissues such as the liver, brain, kidney, pancreas, colon and adrenal gland where it is actively involved in the ATP-dependent extrusion of toxic metabolites and anticancer drugs into the bile, urine and the lumen of the gastrointestinal tract as well as the blood [41]. P-gp plays a central role in maintaining epithelial and endothelial cell barrier function and therefore is overexpressed in the blood–brain barrier, blood-CSF barrier, blood-testis barrier as well as the maternal–foetal barrier in the placenta [42–44]. P-gp was also found in haematopoietic stem cells and lymphocytes, suggesting a role in protecting them from endogenous and exogenous toxicants [45, 46]. P-gp is also overexpressed in various tumour cells, mostly in colon, renal, breast, ovarian and adrenal carcinomas [19, 46, 47]. Furthermore, selection with structurally and mechanistically distinct cytotoxic drugs has been previously shown to provoke P-gp overexpression due to either stable gene amplification or transcriptional activation, rendering these malignant cells resistant to multiple chemotherapeutics. For example, treatment with the following cytotoxic drugs: vincristine [48], doxorubicin [49], mefloquine [50] and colchicine triggered P-gp overexpression in tumour cells [51]. In contrast, some compounds have been shown to reduce P-gp expression including curcumin [52], achieving the opposite result, suppressing MDR of tumour cells to drugs which are P-gp transport substrates.

Expulsion of LysoTracker and MitoTracker Red from P-gp-expressing cells leads to markedly reduced accumulation of these fluorescent markers in their target organelles, hence significantly decreasing organelle fluorescence. The ability to fluorescently label and evaluate quantitative and qualitative features of lysosomes or mitochondria in P-gp-overexpressing cells would be seriously limited or completely abolished. In that case, markedly reduced fluorescence will not be a true indication of the cellular lysosome or mitochondria content and/or function, but rather a result of the viable fluorescent markers being extruded from the cells via P-gp. Thus, the staining of P-gp-expressing cells with these molecular markers may lead to erroneous interpretation of results. The use of LysoTracker dyes in flow cytometry for the evaluation of autophagy that was recently suggested [11, 12] might also lead to misinterpretation of results if fluorescent dyes that are P-gp substrates are used for this aim in P-gp-expressing cells. In such experiments, P-gp-expressing cells will appear to display lower levels of autophagy when in fact the reduced staining is due to P-gp-mediated extrusion of the LysoTracker dye. Such misinterpretation of results might lead, for example, to false conclusions regarding the mechanism underlying anticancer resistance in various cancer cells. Combining these facts with the broad expression of P-gp in various tissues and MDR cancer cell lines and tumour cell lines suggests that P-gp-expression must be seriously taken into consideration when staining cells with LysoTracker and MitoTracker Red, if quantification and functionality of organelle fluorescence is of importance for the interpretation of the data.

While P-gp is predominantly located on the plasma membrane, several studies have also found P-gp to also be localized in the membranes of intracellular organelles including lysosomes [53–55]. A recent study revealed that P-gp may be localized in the lysosomal membrane and contribute to MDR by actively sequestering drugs into lysosomes, thus preventing them from reaching their cellular target [56]. In the context of the current study, lysosomal membrane-targeted P-gp is expected to achieve the opposite effect of plasma membrane P-gp by actively transporting LysoTracker into lysosomes, as opposed to the dye’s primary mechanism of accumulation via simple diffusion and lysosomal accumulation through cation trapping. The latter possibility will enhance LysoTracker fluorescence, hence creating an additional pathway for the misinterpretation of quantitative data. Thus, not only the expression level of P-gp has to be taken into account when staining cells with LysoTracker and MitoTracker Red, if quantification and functionality of organelle fluorescence is of importance for the interpretation of the data.
consideration when staining cells with MitoTracker Red or MitoTracker Red but also its subcellular localization.

While our results demonstrate that it is possible to overcome P-gp-mediated extrusion of LysoTracker or MitoTracker Red from cells using P-gp transport inhibitors such as tariquidar, the inhibition of P-gp might be partial resulting in unreliable quantitative and analytical data. We thus suggest testing of the cells under study for P-gp expression prior to their testing with MitoTracker or MitoTracker Red and choosing different tumour cell lines or different molecular stains if P-gp expression is detected.

Our findings reveal that MitoTracker Red was a much superior P-gp transport substrate than LysoTracker Red; this is strongly supported by the fact that human lung carcinoma SW-1573/2R160 cells with low P-gp overexpression readily expelled MitoTracker Red but failed to extrude LysoTracker Red. While these two fluorescent organelle probes are polyaromatic hydrophobic compounds, a close examination of their structures raises plausible biophysical explanations for this remarkable difference between MitoTracker Red as a bona fide P-gp transport substrate and LysoTracker Red being a relatively poor P-gp substrate. MitoTracker Red is a hydrophobic compound (Log P = 1.15) [14, 56] containing a large planar quinolizino quinoline ring structure in which two nitrogens are positively charged at physiological pH (Fig. 1), where the chloromethylphenyl group is also contained within this planar structure. In this respect, we have previously shown that such large planar hydrophobic aromatic structures, including anticancer drugs with positive charge(s), traverse the plasma membrane relatively slowly via a flip-flop mechanism [56–61]. This slow flip-flop involves an initial insertion of the drug in the outer leaflet of the plasma membrane with the positively charged residues protruding to the extracellular milieu; this is followed by a relatively slow flip-flop into the cytoplasmic membrane leaflet (Fig. 6A). Previous studies have shown that the residence half-life times in the outer leaflet of the membrane for various polyaromatic anthracenes can range from 6 sec. for idarubicin to as long as 6 min. for mitoxantrone. Thus, as P-gp can readily recognize polyaromatic hydrophobic structures with a slight cationic charge and as P-gp displays a remarkable transport substrate turnover number of 900 per second [14, 30, 56], it will have ample time to bind MitoTracker Red within the lipid bilayer and efficiently extrude it. In contrast, LysoTracker Red is a much smaller hydrophobic molecule (Log P = 2.1) [5, 10, 56] containing a tri-pyrrole core as well as a highly flexible aliphatic propanamidato side chain, which harbours a terminal nitrogen that may or may not be positively charged at physiological pH. Consequently, the expected residence time of this relatively small hydrophobic molecule (that is not necessarily charged at physiological pH) in the plasma membrane is expected to be rather short, hence leaving no realistic membrane residence time for substrate binding and extrusion by P-gp.

These considerations of P-gp transport substrate recognition, demonstrated herein by the differences between LysoTracker Red and MitoTracker Red structures and the influence of these structural differences on the efficiency of P-gp-mediated extrusion of these fluorescent dyes from P-gp-expressing cells, bear important implications for the future design of anticancer drugs. From a pharmacological perspective, when designing novel small molecule hydrophobic anticancer drugs, one should avoid the introduction of positively charged residues and/or other moieties that may enhance plasma membrane residence time, hence leaving for P-gp ample time to recognize and efficiently extrude such novel chemotherapeutics, resulting in cancer MDR. Consistently, previous studies have shown that acceleration of passive drug transbilayer movement across the plasma membrane by local anaesthetics markedly abolished the ability of P-gp to recognize and extrude bona fide P-gp substrates, thus overcoming MDR [56, 62]. Another important possible implication of the current study is that these fluorescent probes could be used in a bioassay for high-throughput drug screening protocols that would facilitate the development of potent P-gp transport inhibitors known as MDR chemosensitizers. Thus, large chemical libraries could be readily screened in P-gp-overexpressing cells while searching for efficient restoration of MitoTracker Red fluorescence, which would be used as an indication for successful inhibition of P-gp efflux activity, thus facilitating the development of MDR chemosensitizers for effective reversal of P-gp-mediated cancer MDR, which continues to be a major hindrance to curative chemotherapy of various human malignancies.

Conflict of interests

The authors confirm that there is no conflict of interests.

References

1. Strouse JJ, Ivnitski-Steele I, Waller A, et al. Fluorescent substrates for flow cytometric evaluation of efflux inhibition in ABCB1, ABCC1, and ABCG2 transporters. Anal Biochem. 2013; 437: 77–87.
2. Marques-Santos LF, Oliveira JG, Maia RC, et al. MitoTracker green is a P-glycoprotein substrate. Biosci Rep. 2003; 23: 199–212.
3. Litman T, Brangi M, Hudson E, et al. The multidrug-resistant phenotype associated with overexpression of the new ABC half-transporter, MXR (ABCG2). J Cell Sci. 2000; 113: 2011–21.
Chakote B. Labeling lysosomes in live cells with LysoTracker. Cold Spring Harb Protoc. 2011; 2011: pdb prot5571.

Duvuri M, Gong Y, Chatterji D, et al. Weak base permeability characteristics influence the intracellular sequestration site in the multidrug-resistant human leukemic cell line HL-60. J Biol Chem. 2004; 279: 32367–72.

Alce TM, Popik W. APOBEC3G is incorporated into virus-like particles by a direct interaction with HIV-1 Gag nucleocapsid protein. J Biol Chem. 2004; 279: 34083–8.

Adar Y, Stark M, Bram EE, et al. Imidazooacidine-dependent lysosomal photodestruction: a pharmacological Trojan horse approach to eradicate multidrug-resistant cancers. Cell Death Dis. 2012; 3: e293.

Yogalingam G, Pendergast AM. Abl kinases regulate autophagy by promoting the trafficking and function of lysosomal components. J Biol Chem. 2008; 283: 35941–53.

Zhitoimirsky B, Assaraf YG. Lysosomal sequestration of hydrophobic weak base chemotherapeutics triggers lysosomal bio-destruction: a pharmacological Trojan horse approach to eradicate multidrug-resistant cancers. Cell Death Dis. 2012; 3: e293.

Kazmi F, Hensley T, Pope C, et al. Lysosomal sequestration (trapping) of lipophilic amine (cationic amphiphilic) drugs in immortalized human hepatocytes (FaZN-4 cells). Drug Metab Dispos. 2013; 41: 897–905.

Chikte S, Panchal N, Warnes G. Use of LysoTracker dye: a flow cytometric study of autophagy. Cytometry A. 2014; 85: 169–78.

Warnes G. Flow cytometric assays for the study of autophagy. Methods. 2015; 82: 21–8.

Poot M, Zhang YZ, Kramer JA, et al. Analysis of mitochondrial morphology and function with novel fixable fluorescent stains. J Histochem Cytochem. 1996; 44: 1363–72.

Chyay W, Zhang DY, Lippord SJ, et al. Reaction-based fluorescent sensor for investigating mobile Zn2+ in mitochondria of healthy versus cancerous prostate cells. Proc Natl Acad Sci USA. 2014; 111: 143–8.

Sansenwal P, Yen B, Gahl WA, et al. Mitochondrial autophagy promotes cellular injury in nephropathic cystinosis. J Am Soc Nephrol. 2010; 21: 272–83.

Gislon PR, Yu X, Hereth D, et al. Two Dictyostelium orthologs of the prokaryotic cell division protein FtsZ localize to mitochondria and are required for the maintenance of normal mitochondrial morphology. Eukaryot Cell. 2003; 2: 1315–26.

Spelbrink JN, Li FY, Tiranti V, et al. Human mitochondrial DNA deletions associated with mutations in the gene encoding Twinkle, a phage T7 gene 4-like protein localized in mitochondria. Nat Genet. 2001; 28: 223–31.

Poot M, Gibson LL, Singer VL. Detection of apoptosis in live cells by MitoTracker red CMXRos and SYTO dye flow cytometry. Cytometry. 1997; 27: 558–64.

Dean M, Hamon Y, Chiminì G. The human ATP-binding cassette (ABC) transporter superfamily. J Lipid Res. 2001; 42: 1007–17.

Locher KP. Mechanistic diversity in ATP-binding cassette (ABC) transporters. Nat Struct Mol Biol. 2016; 23: 487–93.

Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer. 2002; 2: 48–58.

Ambudkar SV, Kimchi-Sarfaty C, Sauna ZE, et al. P-glycoprotein: from genomics to mechanism. Oncogene. 2003; 22: 7468–85.

Riordan JR, Ling V. Purification of P-glycoprotein from plasma membrane vesicles of Chinese hamster ovary cell mutants with reduced cholécyt permeability. J Biol Chem. 1979; 254: 12701–5.

Juliano RL, Ling V. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochem Biophys Acta. 1976; 455: 152–62.

Aller SG, Yu J, Ward A, et al. Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. Science. 2009; 323: 1718–22.

Ramachandra M, Ambudkar SV, Chen D, et al. Human P-glycoprotein exhibits reduced affinity for substrates during a catalytic transition state. Biochemistry. 1998; 37: 5010–9.

Lam FC, Liu R, Lu P, et al. beta-Amyloid efflux mediated by p-glycoprotein. J Neurochem. 2001; 76: 1121–8.

Gottesman MM, Pastan I. Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem. 1993; 62: 385–427.

Assaraf YG, Borgnia MJ. Probing the interaction of the multidrug-resistance phenotype with the polypeptide ionophore gramicidin D via functional channel formation. Eur J Biochem. 1994; 222: 813–24.

Borgnia MJ, Eytan GD, Assaraf YG. Competition of hydrophobic peptides, cytotoxic drugs, and chemosensitizers on a common P-glycoprotein pharmacophore as revealed by its ATPase activity. J Biol Chem. 1996; 271: 3163–71.

Hoffmeyer S, Burk O, von Richter O, et al. Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity in vivo. Proc Natl Acad Sci USA. 2000; 97: 3473–8.

Sharam FJ. ABC multidrug transporters: structure, function and role in chemoresistance. Pharmacogenomics. 2008; 9: 105–27.

Iei I. Functional significance of genetic polymorphisms in P-glycoprotein (MDR1, ABCB1) and breast cancer resistance protein (BCRP, ABCG2). Drug Metab Pharmacokinet. 2012; 27: 85–105.

Murota Y, Tabo K, Taga T. Requirement of ABC transporter inhibition and Hoechst 33342 dye deprivation for the assessment of side population-defined C6 glioma stem cell metabolism using fluorescent probes. BMC Cancer. 2016; 16: 847.

van Triest B, Pinedo HM, Tellmann F, et al. Cross-resistance to antifolates in multidrug resistant cell lines with P-glycoprotein or multidrug resistance protein expression. Biochem Pharmacol. 1997; 53: 1855–66.

Heim S, Lage H. Transcriptome analysis of different multidrug-resistant gastric carcinoma cells. In Vivo 2005; 19: 583–90.

Hooijberg JH, Peters GJ, Assaraf YG, et al. The role of multidrug resistance proteins MRPI, MRP2 and MRP3 in cellular folate homeostasis. Biochem Pharmacol. 2003; 65: 765–71.

Bram EE, Ifergan I, Grimberg M, et al. C421 allele-specific ABCG2 gene amplification confers resistance to the antitumor triazolocacidone C-1305 in human lung cancer cells. Biochem Pharmacol. 2007; 74: 41–53.

Ifergan I, Shafan A, Jansen G, et al. Folate deprivation results in the loss of breast cancer resistance protein (BCRP/ABCG2) expression. A role for BCRP in cellular folate homeostasis. J Biol Chem. 2004; 279: 25527–34.

Weidner LD, Fung KL, Kannan P, et al. Tariquidar Is an Inhibitor and Not a Substrate of Human and Mouse P-glycoprotein. Drug Metab Dispos. 2016; 44: 275–82.

Thiebau F, Tsuruo T, Hamada H, et al. Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. Proc Natl Acad Sci USA. 1987; 84: 7735–8.

Cordon-Cardo C, O’Brien JP, Boccia J, et al. Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues. J Histochem Cytochem. 1990; 38: 1277–87.
43. Leslie EM, Deely RG, Cole SP. Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. *Toxicol Appl Pharmacol*. 2005; 204: 216–37.

44. Cordon-Cardo C, O’Brien JP, Casals D, et al. Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proc Natl Acad Sci USA*. 1989; 86: 695–8.

45. Chaudhary PM, Roninson IB. Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells. *Cell*. 1991; 66: 85–94.

46. Chaudhary PM, Mechetner EB, Roninson IB. Expression and activity of the multidrug resistance P-glycoprotein in human peripheral blood lymphocytes. *Blood*. 1992; 80: 2735–9.

47. Leonard GD, Polgar O, Bates SE. ABC transporters and inhibitors: new targets, new agents. *Curr Opin Investig Drugs*. 2002; 3: 1652–9.

48. Anderle P, Niederer E, Rubas W, et al. P-Glycoprotein (P-gp) mediated efflux in Caco-2 cell monolayers: the influence of culturing conditions and drug exposure on P-gp expression levels. *J Pharm Sci*. 1998; 87: 757–62.

49. Slovak ML, Hoeltge GA, Dalton WS, et al. Pharmacological and biological evidence for differing mechanisms of doxorubicin resistance in two human tumor cell lines. *Cancer Res*. 1988; 48: 2793–7.

50. Peel SA, Bright P, Yount B, et al. A strong association between mefloquine and halofantrine resistance and amplification, overexpression, and mutation in the P-glycoprotein gene homolog (ptfndr) of Plasmodium falciparum *in vitro*. *Am J Trop Med Hyg*. 1994; 51: 648–58.

51. Ling V, Thompson LH. Reduced permeability in CHO cells as a mechanism of resistance to colchicine. *J Cell Physiol*. 1974; 83: 103–16.

52. Anuchapreeda S, Leechanachai P, Smith MM, et al. Modulation of P-glycoprotein expression and function by curcumin in multidrug-resistant human KB cells. *Biochem Pharmacol*. 2002; 64: 573–82.

53. Ferrao P, Sincock P, Cole S, et al. Intracellular P-gp contributes to functional drug efflux and resistance in acute myeloid leukemia. *Leuk Res*. 2001; 25: 395–405.

54. Molinari A, Calcabrini A, Meschini S, et al. Subcellular detection and localization of the drug transporter P-glycoprotein in cultured tumor cells. *Curr Protein Pept Sci*. 2002; 3: 653–70.

55. Rajagopal A, Simon SM. Subcellular localization and activity of multidrug resistance proteins. *Mol Biol Cell*. 2003; 14: 3389–99.

56. Yamagishi T, Sahni S, Sharp DM, et al. P-glycoprotein mediates drug resistance via a novel mechanism involving lysosomal sequestration. *J Biol Chem*. 2013; 288: 31761–71.

57. Katzir H, Yeheskel-Hayon D, Regev R, et al. Role of the plasma membrane leaflets in drug uptake and multidrug resistance. *FEBS J*. 2010; 277: 1234–44.

58. Regev R, Yeheskel-Hayon D, Katzir H, et al. Transport of anthracyclines and mitoxantrone across membranes by a flip-flop mechanism. *Biochem Pharmacol*. 2005; 70: 161–9.

59. Eytan GD, Regev R, Oren G, et al. The role of passive transbilayer drug movement in multidrug resistance and its modulation. *J Biol Chem*. 1996; 271: 12897–902.

60. Eytan GD, Regev R, Oren G, et al. Efficiency of P-glycoprotein-mediated exclusion of rhodamine dyes from multidrug-resistant cells is determined by their passive transmembrane movement rate. *Eur J Biochem*. 1997; 248: 104–12.

61. Stark M, Assaraf YG. Structural recognition of tubulysin B derivatives by multidrug resistance efflux transporters in human cancer cells. *Oncotarget*. 2017; 8: 49973–87.

62. Regev R, Katzir H, Yeheskel-Hayon D, et al. Modulation of P-glycoprotein-mediated multidrug resistance by acceleration of passive drug permeation across the plasma membrane. *FEBS J*. 2007; 274: 6204–14.