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Riboflavin concentration within ABCG2-rich extracellular vesicles is a novel marker for multidrug resistance in malignant cells

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ABSTRACT

We have previously shown that overexpression of the multidrug resistance (MDR) efflux transporter ABCG2 in the membrane of novel extracellular vesicles that are confined to breast cancer cell–cell attachment zones confers mitoxantrone resistance and mediates a marked intravesicular concentration of an unknown endogenous green fluorescent compound (I. Ifergan, G.L. Scheffer, Y.G. Assaraf, Novel extracellular vesicles mediate an ABCG2-dependent anticancer drug sequestration and resistance, Cancer Res. 65 (2005) 10952–10958). Here we identified the latter as riboflavin (vitamin B2) and further demonstrated that the marked intravesicular concentration of riboflavin in ABCG2-overexpressing breast and lung cancer cells tightly correlates with the extent of ABCG2 overexpression and its differential localization to the vesicular membrane and not to the plasma membrane surrounded by growth medium. We hence propose that the ABCG2-dependent concentration of riboflavin in these intercellular compartments may serve as a novel, sensitive, and non-cytotoxic (i.e. based on vitamin accumulation) functional marker for the quantification of the levels of MDR mediated by ABCG2-rich extracellular vesicles in multiple malignant cells.

Members of the ATP-Binding Cassette (ABC) transporters including P-glycoprotein (Pgp/ABCB1), multidrug resistance (MDR) proteins (MRPs/ABCC) as well as breast cancer resistance protein (BCRP/ABCG2) function as ATP-dependent drug efflux transporters, which form a unique defense network against multiple chemotherapeutic drugs as well as endogenous and exogenous cellular toxicants [1]. Upon overexpression in the plasma membrane, these MDR efflux transporters confer upon cancer cells MDR phenomena. We have recently shown that overexpression of ABCG2 in the membrane of novel extracellular vesicles of breast cancer MCF-7/MR cells confers resistance to the anticancer drug mitoxantrone and mediates the dramatic intravesicular concentration of an unknown endogenous fluorescent compound [2]. Hence, the initial aim of the current study was to identify this endogenous fluorescent compound which may serve as a sensitive and quantitative functional marker for the presence and extent of MDR mediated by ABCG2-rich extracellular vesicles in tumor cells. Towards this end, we first evaluated the physiological role, chemical properties as well as the fluorescent characteristics of naturally occurring ABCG2 substrates including flavonoids [3–6], dietary toxic molecules such as the carcinogen PhIP [6–8], the chlorophyll derivative pheophorbide A [6,9,10], porphyrins [6,9,11], sulfated estrogens [6,12], and folic acid [13–16]. In contrast to the detoxifying role of ABCG2 in adult humans [7,17,18], ABCG2 expressed in the murine mammary gland during lactation mediates the extrusion of various drugs, toxins, and carcinogens into milk and thus exposes the suckling newborn to xenotoxins [18]. This apparent paradox between the detoxifying (i.e. protective) and contaminating effects of ABCG2 in the breast-feeding newborn has been partially resolved by a recent finding that ABCG2 secretes riboflavin (vitamin B2) into milk, thereby supplying the newborn with this important micronutrient [19]. Milk, dairy products, cereals, meat (especially offal), fatty fish as well as certain dark-green vegetables are good sources of riboflavin [19,20]. Once taken up into cells presumably via the recently cloned riboflavin transporter (RFT1) [21], riboflavin is converted to the essential coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). FMN and FAD serve as electron carriers in a wide spectrum of key enzymatic redox reactions including bioenergetic metabolism [19,20]. Based upon our original observation of the FITC like fluorescence properties (i.e. excitation and emission at 490 and 520 nm, respectively) shared by riboflavin and the intravesicular fluorescence [2], we hypothesized that this vitamin may be responsible for the intense green autofluorescence observed in ABCG2-rich extracellular vesicles in MDR cells with ABCG2 overexpression [2]. Hence, we here demonstrate an ABCG2-dependent riboflavin concentration within extracellular vesicles thereby resulting in an intense green autofluorescence of riboflavin in the lumen of these intercellular compartments. Using breast cancer MCF-7/MR and non-small cell lung cancer A549/K1.5 cell lines with ABCG2-overexpression but distinct cellular ABCG2 localization, we demonstrate

Abbreviations: MDR, multidrug resistance; BCRP, breast cancer resistance protein; FTC, fumitremorgin C; PBS, Phosphate-buffered saline.

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that riboflavin concentration within extracellular vesicles is a sensitive marker for both the presence of functional ABCG2-dependent extracellular vesicles as well as for the cellular localization and activity of ABCG2. We thus propose that the ABCG2-dependent concentration of riboflavin within these intercellular compartments may serve as a novel, sensitive, and non-cytotoxic (i.e. based on vitamin accumulation) functional marker for the quantification of the levels of MDR mediated by ABCG2-rich extracellular vesicles in multiple malignant cells.

Materials and methods

Chemicals

Mitoxantrone hydrochloride was from Cyanamid of Great Britain Ltd. (Gosport, Hampshire, UK). Fumitremorgin C (FTC) was kindly provided by Dr. S.E. Bates, National Cancer Institute, Bethesda, MD. Triazoloacridone C-1305 was synthesized by Dr. B. Horowska and Dr. M. Konieczny at the Department of Pharmacological Technology and Biochemistry, Gdansk University of Technology, Poland.

Tissue culture

MCF-7 human breast cancer cells and their mitoxantrone-resistant MCF-7/MR [22] subline (kindly provided by Dr. S.E. Bates, National Cancer Institute, Bethesda, MD) were grown as monolayers in RPMI-1640 medium as previously described [14,15]. Specifically, MCF-7/MR cells were pulsed with 100 nM mitoxantrone every two weeks for a duration of 3 days. All subsequent experiments were initiated after 4 days of incubation with mitoxantrone-free growth medium. Human A549 non-small cell lung cancer cells were grown under monolayer conditions in RPMI-1640 medium (Invitrogen™-GIBCO® Carlsbad, California) containing 10% fetal calf serum, 2 mM glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin (Biological Industries, Beth-Haemek, Israel) in a humidified atmosphere of 5% CO_2. C-1305-resistant non-small cell lung cancer A549/K1.5 cells were pulsed with 1.5 μM triazoloacridone C-1305 every two weeks for a duration of 3 days. All subsequent experiments were initiated after 4 days of incubation with a C-1305-free growth medium.

Determination of ABCG2-dependent riboflavin concentration in extracellular vesicles of ABCG2-overexpressing MCF-7/MR and A549/K1.5 cells

MCF-7/MR and A549/K1.5 cells were trypsinized and washed three times with riboflavin-free growth medium (Invitrogen™-GIBCO® Carlsbad, California). Then, 2×10^4 cells/well were seeded onto 6-well plates in riboflavin-free growth medium. As a positive control of the endogenous autofluorescent compound in the extracellular vesicles we used cells grown in riboflavin-containing growth medium. Likewise, as a negative control of ABCG2-dependent riboflavin concentration in extracellular vesicles we used cells grown in riboflavin-containing growth medium in the presence of 5 μM FTC, a specific ABCG2 inhibitor. Following 7 days of incubation, monolayer cells were washed and incubated with riboflavin-free growth medium for bright field and FITC-like modes analyses at Leica microscope.

Estimation of the intravesicular concentration levels of riboflavin

MCF-7/MR cells and A549/K1.5 cells were trypsinized and seeded (i.e. 2×10^4cells) onto wells of 6-well plates in riboflavin-containing (i.e. 0.53 μM) growth medium. Following 7 days of incubation, wells were washed with riboflavin-free growth medium and then incubated with 15-μl aliquots of standard solutions containing increasing riboflavin concentrations in 20 mM HEPES covered by glass coverslips. In order to generate a calibration curve, photographs were taken at random locations using a bright field and FITC-like mode analyses using a Leica microscope (Fig. 2B). Photographs were then transformed to a gray scale format and analyzed individually by scanning densitometry using the program "TINA" (version 2.10g). The densitometric levels of each riboflavin concentration was obtained from the cell-free areas in order to generate the calibration curve used to calculate the intravesicular concentration of riboflavin in the extracellular vesicles after subtraction of background levels (i.e. densitometric level of areas free of cells in riboflavin-free photographs). The results are based on analysis of a total number of ~60 extracellular vesicles for each cell line.

Western blot analysis of ABCG2 expression

To examine the expression of ABCG2 in the various cell lines, semi-quantitative Western blot analysis was preformed with rat-anti-ABCG2 monoclonal antibody BXP-53 as described previously [14,15,23]. Likewise, an affinity-purified rabbit polyclonal antiserum to the alpha subunit of Na^+/K^+ ATPase (anti-KETTY, at 1:3000 dilution, kindly provided by Prof. S.J. Karlish, Department of Biological Chemistry, The Weizmann Institute of Science, Rehovot, Israel) was used as an indication of loading differences.

Immunohistochemical and immunofluorescence microscopy studies of MCF-7/MR and A549/K1.5 cells with anti-ABCG2 antibodies

Immunohistochemistry studies. MCF-7/MR and A549/K1.5 cells were trypsinized and seeded (i.e. 1×10^4 cells) onto 24-well plates. Following 7 days of incubation, an immunohistochemical analysis with anti-ABCG2 monoclonal antibody BXP-53 (1:100 dilution) was performed as described previously [15]. Cells were then examined with a Leica microscope at a bright field mode.

Immunofluorescence studies. Cells (1×10^4) were seeded in 24-well plates (2 ml medium/well) on sterile glass coverslips and incubated for 7 days at 37 °C. Then, cells underwent an identical staining protocol as described above (see *Immunohistochemistry studies*) with the following modifications: (1) BXP-21 (at a dilution of 1:100, generously provided by Prof. R.J. Scheper and Dr. G.L. Scheffer, VU University Medical Center, Amsterdam, The Netherlands) served as the primary ABCG2-specific monoclonal antibody. (2) FITC-conjugated Donkey anti-mouse IgG (1:100 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA) served as the secondary antibody. (3) Cell nuclei were stained with the DNA dye DAPI (Sigma–Aldrich) which was added at a final concentration of 0.5 μg/ml during the incubation with the secondary antibody. After four washes with PBS (each with 2 ml), the coverslips were mounted onto glass slides using Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL). Slides were then examined using a Leica immunofluorescence microscope.

Statistical analysis

We used the non-parametric Mann–Whitney *U* test to compare the significance of the difference between two populations for a certain variable.

Results

ABCG2-dependent riboflavin concentration in extracellular vesicles of ABCG2 overexpressing breast cancer MCF-7/MR and non-small cell lung cancer A549/K1.5 cells

Under standard growth conditions in riboflavin-containing RPMI-1640 medium (i.e. routinely containing 0.5 μM riboflavin),

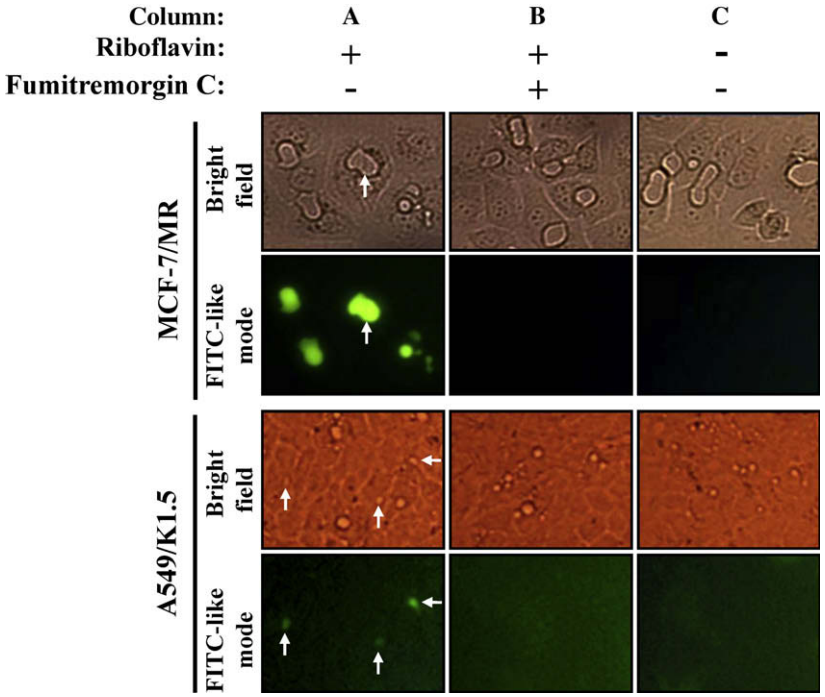

Fig. 1. ABCG2-dependent riboflavin concentration in extracellular vesicles of ABCG2-overexpressing breast cancer MCF-7/MR and non-small cell lung cancer A549/K1.5 cells: MCF-7/MR and A549/K1.5 cells were grown for one week in the following conditions: (A) riboflavin-containing medium (left column), (B) riboflavin-containing medium supplemented with FTC, a specific ABCG2 transport inhibitor (middle column), and (C) riboflavin-free growth medium (right column). Then, random colonies were analyzed under bright field and FITC-like mode using Leica microscope at a magnification of 200× and 400× for MCF-7/MR and A549/K1.5 cells, respectively. Note that the endogenous green fluorescence within extracellular vesicles is denoted by *white arrows*.

the extracellular vesicles were easily identifiable by an endogenous, intense green fluorescence in ABCG2-overexpressing MCF-7/MR and A549/K1.5 cells (Fig. 1: left column). However, this intravesicular fluorescence was completely lost upon cellular growth in the presence of fumitremorgin C (FTC), a specific ABCG2 inhibitor, as well as upon growth in riboflavin-free medium (Fig. 1: middle and right column, respectively).

Quantification of the intravesicular concentration of riboflavin in ABCG2-overexpressing MCF-7/MR and A549/K1.5 cells

Based on a calibration curve obtained with known riboflavin concentrations (e.g. Fig. 2D) and after analyzing a total of ~60 extracellular vesicles for each cell line, the intravesicular concentration of riboflavin was estimated to be as high as 295.0 μM in

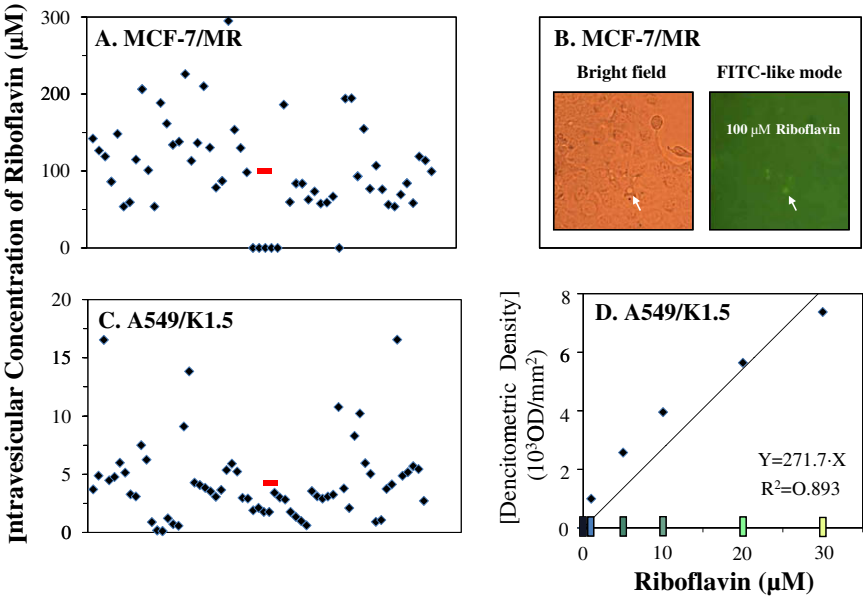

Fig. 2. Intravesicular concentration levels of riboflavin in ABCG2-overexpressing MCF-7/MR and A549/K1.5 cells: The intravesicular concentrations of riboflavin in a total number of ~60 extracellular vesicles for each cell line (i.e. MCF-7/MR and A549/K1.5 cells) were calculated based upon a calibration curve (see Materials and Methods and e.g. Fig. 2D) and presented as a scatter plot shown in (A) and (C), respectively. Riboflavin concentration of each extracellular vesicle examined is represented by a diamond, whereas the average intravesicular concentration of riboflavin for each cell line is marked as a red horizontal bar (A,C). Furthermore, a bright field and a FITC-like mode analysis of MCF-7/MR cells derived immediately after incubation with 100 μM riboflavin demonstrates an augmented green fluorescence (i.e. more than 100 μM riboflavin) in various extracellular vesicles (B, *white arrows*).

MCF-7/MR cells (Fig. 2A) and 16.6 μM in A549/K1.5 cells (Fig. 2C). Hence, the intravesicular concentration of riboflavin in MCF-7/MR and A549/K1.5 cells was, respectively, 557- and ~31-fold higher than that of the culture medium (i.e. 0.5 μM). Likewise, the intravesicular concentration of riboflavin was characterized by a high variability among the individual vesicles and was estimated to be 103.1 ± 62.5 (e.g. Fig. 2B) and 4.3 ± 3.4 μM in MCF-7/MR and A549/K1.5 cells, respectively (Fig. 2A and C, the average is denoted by horizontal bars). Accordingly, we found that the intravesicular concentration of riboflavin was significantly lower in A549/K1.5 cells, when compared with MCF-7/MR cells (*P*-value $<10^{-12}$ as calculated by a non-parametric Mann–Whitney *U* test).

Distinct levels and confinement of ABCG2 in breast cancer MCF-7/MR cells and non-small cell lung cancer A549/K1.5 cells

Western blot analysis revealed 3-fold increased ABCG2 levels in A549/K1.5 cells relative to MCF-7/MR cells (Fig. 3A). Consistently, comparative studies with both immunohistochemical and immunofluorescence microscopy corroborated the higher levels of cellular ABCG2 in A549/K1.5 cells when compared to MCF-7/MR cells (Fig. 3B). However, whereas both cell lines expressed ABCG2 in the membrane of extracellular vesicles, only A549/K1.5 cells expressed this MDR transporter in the plasma membrane surrounded by the growth medium (Fig. 3B: right column).

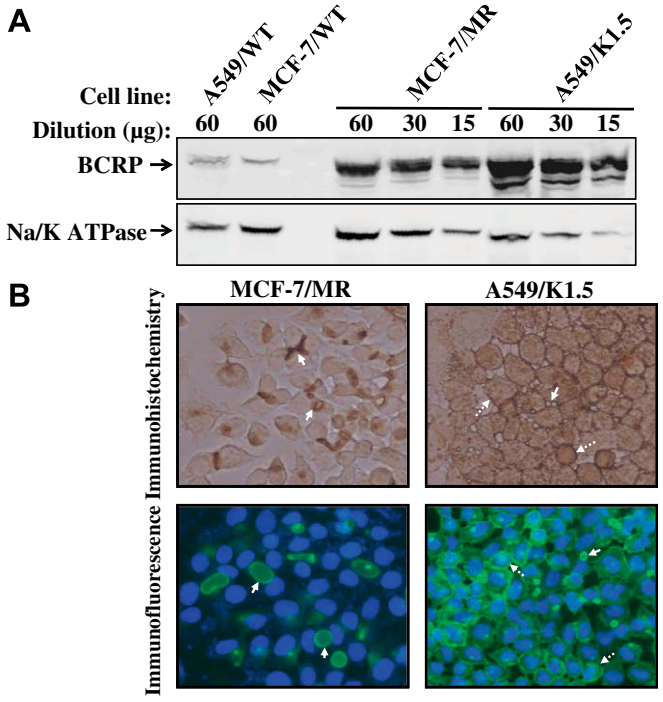

Fig. 3. Levels and confinement of ABCG2 in breast cancer MCF-7/MR cells and non-small cell lung cancer A549/K1.5 cells: (A) ABCG2 expression in MCF-7/MR, A549/K1.5 cells and their parental cell counterparts: ABCG2 levels were determined by semi-quantitative Western blot analysis using ABCG2-specific monoclonal antibody, BXP-53 (upper panel), whereas levels of the internal control Na^+/K^+ ATPase were determined using an affinity-purified rabbit polyclonal antiserum to the alpha subunit of Na^+/K^+ ATPase (lower panel), as described in Materials and Methods. Equal amounts of cellular protein extract (60 μg) were analyzed for each cell line, as protein extracts isolated from the parental cell lines (i.e. MCF-7/WT and A549/WT) served as an indication of normal ABCG2 levels. In addition, two-fold serial dilutions were preformed for the MCF-7/MR and the A549/K1.5 cells (30 and 15 μg, respectively). ABCG2 levels were normalized relative to the Na^+/K^+ ATPase signal by scanning densitometry using the program *TINA* (version 2.10g). (B) Immunohistochemical and immunofluorescence detection of ABCG2 in MCF-7/MR and A549/K1.5 cells: MCF-7/MR and A549/K1.5 cells were fixed with 4% formaldehyde and reacted with an anti-ABCG2 monoclonal antibody, BXP-53. Then, an HRP-conjugated goat anti-rat IgG was added, and brown color development was carried out using the chromogen 3,3′-diaminobenzidine (upper panel). Likewise, we performed immunofluorescence detection of ABCG2 with a FITC-conjugated antibody (green fluorescence, lower panel). Nuclei were counterstained with the DNA dye DAPI (emitting a blue fluorescence). Cells were then examined with a light microscope at 400× magnification for bright field and FITC-like modes analysis using a Leica microscope. The *continuous* white arrows denote the localization of ABCG2 in extracellular vesicles, particularly at the regions of cell–cell attachment zones, whereas the *dashed* white arrows denote the plasma membrane localization of ABCG2 not necessarily at cell–cell attachment zones.

Discussion

Previously we have shown that ABCG2-rich extracellular vesicles were easily identifiable by an intense endogenous green fluorescence which was completely lost upon cellular growth in the presence of the specific ABCG2 inhibitor FTC [2]. Here we hypothesized that the compound emitting this autofluorescence is the B2 vitamin riboflavin which was recently shown to be secreted into milk via ABCG2, thereby supplying the breast-fed newborns with this important micronutrient [19]. Indeed, several lines of evidence establish that riboflavin is the previously unknown compound responsible for the intravesicular green fluorescence in MCF-7/MR and A549/K1.5 cells. First, this endogenous autofluorescence was completely lost upon cellular growth in riboflavin-free growth medium (Fig. 1). Furthermore, the intravesicular fluorescence was completely lost upon cellular growth in the presence of FTC, a specific ABCG2 transport inhibitor (Fig. 1).

Hence, we suggest that the ABCG2-dependent concentration of riboflavin in the lumen of these intercellular vesicles may serve as a sensitive and non-cytotoxic marker for the presence of functional extracellular vesicles in cell lines of distinct tissue origin. Indeed, we herein identified a riboflavin- and ABCG2-dependent intercellular green fluorescence in non-small cell lung cancer A549/K1.5 cells. This fluorescence was confined to cell–cell attachment zones that were occupied by extracellular vesicles (Fig. 1). Importantly, the appearance of extracellular vesicles in cells of diverse tissue origin including breast and lung epithelia suggests that these extracellular vesicles may be present in a broad tissue origin which is not necessarily restricted to breast cancer cells. Hence, a spectrum-wide screening of riboflavin-dependent intercellular green fluorescence in different cultured monolayers of various tumor cell lines of distinct tissue origin is warranted that may pave the way for the identification of extracellular vesicles in multiple neoplasms that may frequently harbor this novel modality of MDR.

Consistent with our previous report of the ~1000-fold concentrative capacity of mitoxantrone in the lumen of extracellular vesicles in MCF-7/MR cells [2], the maximal intravesicular concentration capacity of riboflavin in MCF-7/MR cells here was ~560-fold higher than the concentration of riboflavin in the culture medium (Fig. 2A). Despite the substantially higher levels of ABCG2 in A549/K1.5 cells relative to MCF-7/MR cells (Fig. 3A), the maximal intravesicular concentration of riboflavin in A549/K1.5 cells was only ~31-fold higher than the concentration of riboflavin in the culture medium (Fig. 2C). However, the distinct pattern of cellular localization of ABCG2 revealed a plausible explanation for this apparent discrepancy (Fig. 3B). Hence, we herein present a model in which the plasma membrane confinement of ABCG2 in A549/K1.5 cells in areas surrounded by the growth medium mediates the efflux of riboflavin (Figs. 3B and 4). This ABCG2-dependent efflux may therefore result in decreased intracellular levels of riboflavin and thus decreased rate of ABCG2-dependent riboflavin sequestration within extracellular vesicles (Fig. 4). This model is also consistent with the increased levels of riboflavin concentration in MCF-7/MR cells that completely lack plasma membrane confinement of ABCG2 in areas

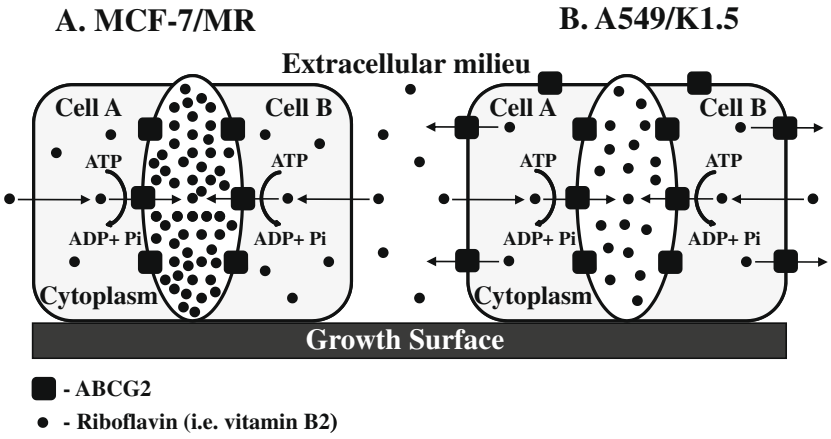

Fig. 4. Modeling of riboflavin transport in MCF-7/MR and A549/K1.5 cells: (A) Modeling of riboflavin transport in MCF-7/MR cells: MCF-7/MR cells lack plasma membrane confinement of ABCG2 in areas surrounded by growth medium and thereby render riboflavin accessible to ABCG2-mediated intravesicular sequestration. (B) Modeling of riboflavin transport in A549/K1.5 cells: the plasma membrane confinement of ABCG2 in A549/K1.5 cells in areas surrounded by the growth medium mediates the efflux of riboflavin. This ABCG2-mediated efflux results in decreased intracellular levels of riboflavin and thus decreased rate of riboflavin sequestration into the extracellular vesicles, when compared to MCF-7/MR cells, even in the presence of ABCG2 in the membrane of the extracellular vesicles.

surrounded by growth medium (Fig. 3B), thereby rendering riboflavin fully accessible to ABCG2-mediated intravesicular sequestration (Fig. 4). Thus, the level of riboflavin concentration in the lumen of extracellular vesicles may readily serve as functional indicator of increased localization of ABCG2 in extracellular vesicles along with decreased levels of this transporter in the plasma membrane surrounded by growth medium. Additionally, this study strongly suggests that A549/K1.5 cells which display resistance to the triazoloacridone C-1305 (i.e. an antitumor agent and a bona fide ABCG2 transport substrate) [23], use two different ABCG2-dependent anticancer drug defense systems; the anticancer drug is first pumped out into the growth medium [23], whereas the drug molecules that evade this initial defense system may be then actively pumped into the extracellular vesicles, thereby resulting in drug sequestration far away from their intracellular target. Interestingly, this study presents two ABCG2-overexpressing cell lines which harbor extracellular vesicles with partially different cellular localization of this transporter (Figs. 3B and 4). Thus, these cell lines may be used to reveal the underlying biochemical signaling pathway(s) responsible for the sorting of ABCG2 into the normal plasma membrane versus plasma membrane which surrounds the extracellular vesicles. In summary, the ABCG2-dependent level of intercellular riboflavin concentration may serve as a sensitive, non-cytotoxic marker for the quantification of MDR that is mediated by ABCG2-dependent extracellular vesicles.

Acknowledgments

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