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Rapid determination of multidrug resistance-associated protein in cancer cells by capillary electrophoresis immunoassay

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ABSTRACT

The adenosine triphosphate (ATP) binding-cassette (ABC) transporters are a superfamily of cellular proteins that have been partly implicated as a cause of multidrug resistance (MDR) in cancer cells. The ABC superfamily consists of P-glycoprotein, multidrug resistance-associated proteins (MRP) and breast cancer-related proteins, of which MRP is of particular interest because of its ability to efflux a broader range of substrates. Since MRP1 is the most prominent member of the MRP family, a simple technique is needed for its quantification. We developed a simple, fast (total analysis time of 3 h) capillary electrophoresis immunoassay (CEIA) for the quantification of MRP1 in cancer cells. MRP1 antibody was labeled with fluorescein isothiocyanate. The labeled antibody was incubated with the cell lysate for a fixed interval (1 h), after which the cell lysate mixture was directly injected into the capillary to separate the complex of MRP1 and its antibody from free antibody. The noncompetitive CEIA method had a limit of detection of 0.2 nM and a good linear range ($1.7–14.9 \times 10^4$ cells), and was fairly reproducible (RSD < 10%). The results showed that two cell lines, A549 and RDES, expressed MRP1 in the absence of doxorubicin (DOX), with A549 registering a higher expression. Compared to DOX-free cancer cells, there was an acceleration of MRP1 expression during the 12 h-exposure to DOX, after which the level of expression remained nearly constant as the intracellular accumulation of DOX decreased. The results obtained in this work indicate that the developed CEIA method is useful for relative quantification of MRPs in cancer cells.

1. Introduction

Chemotherapy treatment of many types of cancers is rendered ineffective due to intrinsic or acquired multidrug resistance (MDR), which is partly induced by multidrug transporter proteins such as the adenosine triphosphate (ATP) binding-cassette (ABC) and lung resistance-related proteins [1–3]. These multidrug transporter proteins actively efflux drugs out of the cells, thereby reducing their intracellular concentration and leading to MDR. The ABC superfamily constitutes the bulk of the multidrug transporter proteins, and consists of three main families: multidrug resistance-associated protein (MRP), P-glycoprotein, and breast cancer-related proteins [1]. Although it has been less thoroughly investigated than P-glycoprotein, MRP can efflux not only cationic and neutral hydrophobic compounds, but also anionic conjugates of sulfates, glutathione, and glucuronic acid. MRP is made up of several subfamilies including MRP1, MRP2, MRP3, MRP4, and MRP5. Because of the role of MPR1 in conferring MDR in tumors [4], along with its wide occurrence in the human body, quantification of MRP1 is extremely important.

Absolute and relative quantification of the protein transporters has been reported. While absolute quantification of these transporter proteins is most useful, it is difficult, time consuming, and expensive, primarily because standards must be synthesized, purified, and identified prior to quantification by one of the analytical methods, e.g., HPLC [5,6]. Most methods, however, are based on relative quantification, in which the proteins are analyzed by various techniques without using standards. Methods of transporter protein quantification that have been studied include PCR [7–9] (RT-PCR, real time RT-PCR), Western blotting [10,11], flow cytometry [12,13], and electrochemical immunoassay [14]. Western blotting is not only semi-quantitative but also time consuming and requires large sample sizes. The main disadvantages of flow cytometry are its expensive instrumentation and difficulty in the determination of transporter proteins localized at cell organelles, since flow cytometry only measures the transporter

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proteins located at the cell surface. PCR techniques require a longer analysis time for separation, detection, and accurate quantification, and may suffer from contamination of the probe, which may lead to false positives [15].

Although ABC transporter proteins are generally thought to mediate drug efflux at the plasma membrane [16–18], some studies have shown that these proteins are localized in cell organelles like the nucleus [19,20]. Because the transporter proteins could be localized anywhere in the cells, it is more useful to determine the total intracellular amount of the transporter protein after carrying out cell lysis. Such determinations are more suitably carried out by capillary electrophoresis immunoassay (CEIA). Indeed CEIA may address some of the shortcomings of the established methods such as ELISA, Western blotting, and flow cytometry because it is easy to automate, requires smaller sample sizes and shorter analysis time, has simple procedures, and is capable of multi-analyte analysis [21]. CEIA in either competitive [22,23] or noncompetitive [24] formats, may utilize antibody [22], enzymes [25] or aptamers [26,27] as ligand to interact with antigens to form complexes in highly complicated matrices. Since the pioneering works by Nielsen et al. [28], CEIA has found application in the determination of wide range of analytes including toxins [29], drugs and metabolites [30], hormones [31], peptides [32], and proteins [33]. While most CEIA investigations of proteins have focused on lower molecular weight proteins (10–80 kD), reports on the determination of higher molecular weight proteins, like ABC transporter proteins (170–190 kD) in cells are few. It is worth noting that even CEIA reports of the most extensively studied ABC transporter-protein, P-glycoprotein, are rare.

In the present study, a simple, noncompetitive CEIA method for the relative quantification of MRP1 was developed. Laser-induced fluorescence (LIF) was used for detection of the transporter protein in order to solve the problem of low sensitivity inherent in the capillary electrophoresis (CE) technique. Since baseline resolution of complex and antibody is necessary for this method, antibody instead of enzymes or aptamers was employed because the smaller size of the two ligands will lead to poor resolution between the complex and free ligand for bulky proteins such as MRP1. The method involved reacting cell lysate with an excess of the labeled anti-MRP1 antibody and adding an internal standard, followed by immediate injection of the unincubated mixture into the CE system to obtain the antibody peak before the immunological reaction. After two or three swift, consecutive runs, the cell lysate mixture was incubated, after which more CE runs were made to obtain peaks for the free antibody and formed immune complex. The amount of the formed immune complex was used to determine the amount of protein contained in the cell lysate. It should be noted that no purification of the antibody was necessary, as quantification of the protein is based on the immune complex and not the post-incubation amount of the antibody. This method was used to compare the levels of MRP1 expressed in cancer cells A549 and RDES.

2. Materials and methods

2.1. Materials

Sodium tetraborate decahydrate, glycine, tricine, doxorubicin (DOX, in hydrochloride form), absolute ethanol, rhodamine B, hydrochloric acid, sodium fluorescein, and Tris were purchased from Wako Pure Chemicals (Osaka, Japan). Monoclonal anti-MRP1 (Clone QCRL-4, Purified Mouse Immunoglobulin, Product Number M9192), sodium dodecylsulfate (SDS-electrophoresis grade), sodium taurodeoxycholate (STDC) hydrate, and (2-hydroxypropyl)-γ-cyclodextrin, were obtained from Sigma–Aldrich (St. Louis, MO, USA). A Fluorescein Labeling Kit-NH_2 and EDTA were obtained from Dojindo (Kumamoto, Japan). Sodium chloride was obtained from Chameleon Reagents (Osaka, Japan). A bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Biotechnology (Rockford, IL, USA). Lung cancer cells, A549, were purchased from the Health Science Research Resources Bank (Osaka, Japan). Human Ewing's family tumor cell line (RDES) was obtained from the American Type Culture Collection (VA, USA).

Stock solutions of trypsin-EDTA (0.05%), RPMI and DMEM media, and DPBS (1X) were purchased from Invitrogen (Grand Island, NY, USA). All solutions were prepared in pure 18-MΩ MilliQ water (Millipore SA, Molsheim, France). A stock solution of DOX (200 μM) was prepared in MilliQ water, stored in opaque containers and kept refrigerated at 4 °C. The migration solution consisted of sodium tetraborate (120 mM of borate), glycine (50 mM), and tricine (50 mM) adjusted to pH 8.9. The preparation of the migration solution for DOX measurement has been described elsewhere [34].

2.2. Treatment of cells with DOX

Prior to treatment with DOX for a fixed time interval (12 h or 24 h), the cells (A549 or RDES) were washed thrice with DPBS and separated into 3.5-cm petri dishes. The cells in the dishes were cultured until they covered 90–100% of the bottom surface area of the dish. Thereafter, fresh culture media with and without DOX were added to the dishes to prepare DOX-free and DOX-treated (500 nM) cells. After addition of the appropriate culture medium, the cells were incubated at 37 °C in 5% CO_2 for either 12 h or 24 h. Subsequently, the cells were lifted by adding 200 μL of trypsin-EDTA, suspended by adding 800 μL of DPBS, and then transferred into a microvial, where they were washed (twice or thrice) with DPBS, before addition of the cell lysis buffer (400 μL). The cell lysis buffer contains 100 mM NaCl, 20 mM EDTA, 1% (w/v) SDS, and 50 mM Tris–HCl (pH 8). The treatment of cells to obtain lysate and measurement of the total protein content were described earlier [34]. Briefly, the lysis buffer was added to the cells in the microvial. The solution was vortexed to enhance lysis and to make the cell lysate uniform. After complete dissolution of the cells, the cell lysate was sonicated for about 15 min to assist in breaking the long DNA strands, which results in a uniform cell lysate of lower viscosity. The obtained cell lysate was used for antibody binding and protein determination experiments.

2.3. Reaction of cell lysate with antibody

The antibody was labeled with flourescein according to the labeling kit manufacturer's instructions (Dojindo, Kumamoto). The concentration of the labeled antibody was then determined by spectroscopic measurement at 280 and 500 nm. The number of fluorescein molecules tagged with antibody was also calculated according to the labeling kit manufacturer's instructions, using absorbance at 280 and 500 nm. The number was calculated to be 5–7 depending on the concentration ratio of the labeling reagent to the antibody. However, the antibodies tagged with different numbers of fluorescein molecule did not show any difference in the immunological reaction. Therefore, the labeled antibody tagged with 5–7 fluorescein molecules were directly employed for the immunoassay.

In the immunological reaction, a known excess amount of the labeled antibody (30 nM) was added to 60 μL of the sample, followed by the sodium fluorescein (0.125 μM) as internal standard and enough 1x PBS buffer to make 100 μL. Two or three CE-LIF runs were made quickly, before the cell lysate mixture was incubated at 37 °C for 1 h, after which the mixture was directly injected into the capillary for separation by CE-LIF measurement.

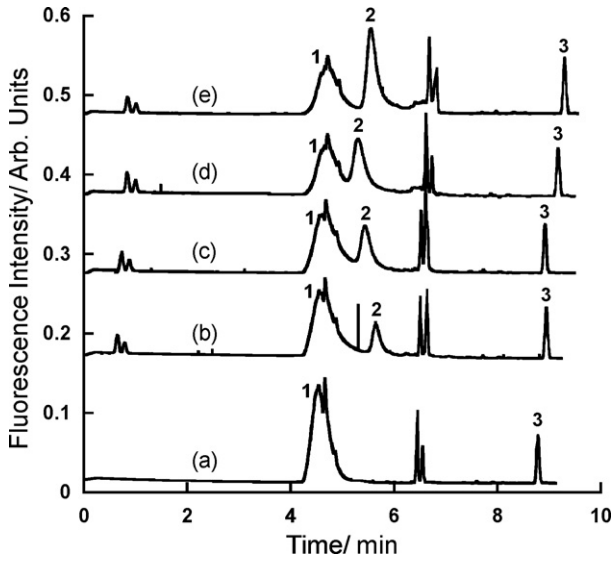

Fig. 1. Formation of the immune complex at different incubation times: (a) 0 min, (b) 11 min, (c) 22 min, (d) 42 min, and (e) 62 min. 1, anti-MRP1; 2, immune complex; 3, fluorescein. Sample: A549 cell lysate treated with DOX for 12 h, incubation temperature 37 °C. Conditions for electrophoresis are given in the text.

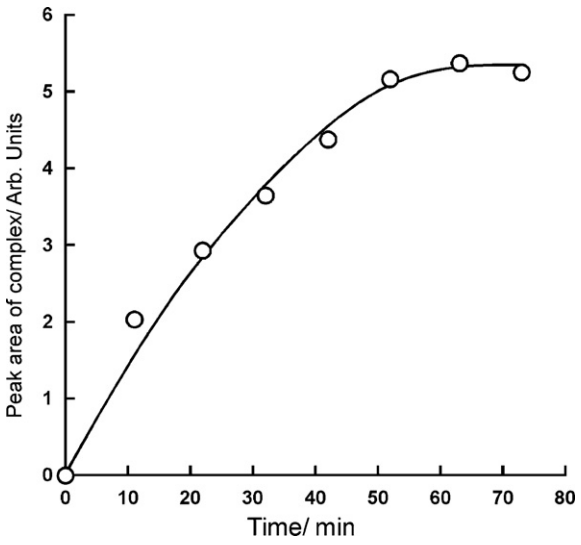

Fig. 2. Kinetic curve of the immune complex formation. Conditions are the same as for Fig. 1.

2.4. CE-LIF measurement

The CE-LIF system used was described previously [34]. Briefly, a custom-made system was assembled in a room with a constant temperature (25 °C). Ordinary fused silica capillaries (50 μm i.d.; 375 μm o.d.; effective length, 30 cm; total length, 40 cm; GL Sciences, Tokyo, Japan) were used in the CE-LIF system. Samples were hydrodynamically injected into the capillary for 10 s by siphoning (the sample vial raised 5 cm above the outlet vial), and a separating voltage (10 kV or 15 kV) was applied using a high voltage power supply (HCZE-30PN0.25, Matsusada Precision Inc., Shiga, Japan). The LIF detection was done using a 488 nm line of an argon ion laser (Stabilite 2017, Spectra-Physics, Inc., CA, USA) as the excitation source. The generated fluorescence was filtered with a notch filter (Edmund Optics Japan, 46564-K, Tokyo, Japan) and collected by a photomultiplier tube (model R3896, Hamamatsu, Shizuoka, Japan) biased at 650 V. The data generated were processed using an in-house Labview program (National Instruments, Austin, TX, USA). The capillary was flushed after every two runs with NaOH (0.1 M) and migration solution for 4 min each.

2.5. Data processing

Pre-incubation electrophoretic measurements were made to determine the peak area corresponding to the initial amount of the antibody ($A_{ab,0}$) and the internal standard ($A_{IS\text{-}pre}$). Post-incubation electrophoretic measurements yielded the peak area corresponding to the complex (A_{comp}) and the internal standard ($A_{IS\text{-}post}$). The peak areas were proportional to the concentrations of the corresponding species. Thus,

$$\frac{C_{ab,0}}{A_{ab,0}/A_{IS\text{-}pre}} = \frac{C_{comp}}{A_{comp}/A_{IS\text{-}post}} \quad (1)$$

where $C_{ab,0}$ and C_{comp} represented the initial concentration of antibody and the concentration of complex produced, respectively.

Under the condition where excess amounts of antibody was added, the complex formed consisted of one antibody and one antigen and the concentration of MRP1 was directly calculated according to Eq. (2):

$$C_{MRP1} = \frac{C_{ab,0}}{A_{ab,0}/A_{IS\text{-}pre}} \times \frac{A_{comp}}{A_{IS\text{-}post}} \quad (2)$$

To correct the concentration of C_{MRP1} for the number of cells, C_{MRP1} was divided by concentration of protein $C_{Protein}$ denoted by the amount of total protein P (mg mL^{-1}), as follows:

$$\frac{C_{MRP1}}{C_{Protein}} = \frac{C_{ab}/(A_{ab}/A_{IS\text{-}pre}) \times A_{comp}/A_{IS\text{-}post}}{P} \quad (3)$$

Using Eq. (3), simple, direct comparison of MRP1 expression in cell lysate is readily accomplished as compared to the more difficult and expensive determination of absolute amounts.

3. Results and discussion

3.1. Method development and kinetics of the complex formation

Noncompetitive CEIA was adopted because of the scarcity of transporter proteins standards (commercial or synthesized). Cell lysates of A549 were employed as samples for optimization of the separation conditions, since it is known that A549 inherently expresses MRP1 [35]. Several migration buffers were tested, including borate (pH 9), MES (pH 7), HEPES (pH 8), CAPS (pH 9.5), and Tris (pH 8.1), but the borate buffer showed the best separation of the antibody and its complex. To control adsorption of both the antibody and the complex on ordinary silica capillary walls, zwitter ionic additives (glycine, tricine) were examined. Borate–glycine (pH 9.0) produced inferior resolution of the two peaks, while borate–tricine exhibited improved peak resolution but suffered peak tailing. Thus, the two zwitter ions were combined to make the migration solution of 50 mM glycine and 50 mM tricine in 120 mM borate buffer (pH 8.9). Variable concentrations (40–150 mM) of the borate buffer were examined and the optimum concentration was found to be 120 mM. The applied voltage was optimized to 10 kV to simultaneously maintain the current below 50 μA and the resolution between the antibody and the complex.

The incubation time for antibody-MRP1 complexation was determined by injecting the mixture of A549 cell lysate and anti-MRP1 at 10 min intervals for a total duration of 73 min. During this period, the mixture was incubated at 37 °C and sample was directly injected into the capillary. Fig. 1 shows the progression of complex formation as the complex peak became increasingly

Table 1
Analytical parameters of the CEIA-LIF method for MRP1 quantification.

Cells/L ($\times 10^4$)	Precision (RSD (%))	
	Intraday	Interday
3.3	6.2	8.18
6.6	5.9	7.16
13.2	5.6	6.61

$n = 7$, LOD (estimated at S/N = 3) = 0.2 nM, Linear range; 1.7–14.9 $\times 10^4$ Cells.

prominent. As seen in Fig. 1, the complex peak appeared only when the cell lysate was mixed with anti-MRP1 followed by incubation. Therefore, the new peak was definitely assigned to the complex. Fig. 2 illustrates the relationship between reaction time and the relative peak area of the complex. The curve in Fig. 2 shows that complex formation was rapid during the first 10–15 min and was nearly complete after about 50 min. This method can, therefore, be used for kinetic investigation of antibody–antigen interaction, as it is possible to directly inject the sample into the capillary at fixed time intervals ($\Delta t > 10$ min) as incubation proceeds. Based on the results in Fig. 2, an incubation time of 60 min was adopted, as the peak area remained nearly constant after 60 min. Although Wang et al. [36] reported improved stability of the complex upon addition of BSA into the cell lysate before adding the antibody, no effect on the stability of either the complex or antibody was observed in this work.

Although the incubation time of 60 min seems to be long for a reaction in a free solution, the kinetics of an immunological reaction is not necessarily fast even in the free solution and is dependent on the type of a target protein. For example, the incubation time of insulin antibody was only 5 min which is a short incubation time [37] whereas protein G needed 30 min of incubation [38] and carcinoembryonic antigen was incubated for 45 min and 60 min with primary and secondary antibodies, respectively [39].

Table 1 summarizes some of the analytical parameters of this CEIA method for MRP1 determination. Compared to the Western blot determination of P-glycoprotein in human colon adenocaranoma cells LS-180 [40], intraday variation in this work was comparable (7.1%), while the interday variation was better than the reported value (17.4%). The LOD obtained was similar to 0.9 nM obtained by competitive CEIA [23], but higher than 5×10^{-12} M determined by noncompetitive IEF [24]. The lower LOD is observed in noncompetitive IEF because the method incorporates a concentration step. It is worth noting that selectivity against other closely related MRPs like MRP2, MRP3 was not tested since the manufacturer of anti-MRP1 antibody indicated that no cross reaction against other MRPs was expected.

Like A549 cells [4,35], RDES cells would be expected to express MRP1, since MRP1 expression has been detected in myeloma samples [41]. Therefore, RDES cell lysates were reacted with labeled anti-MRP1. Fig. 3 shows a typical separation of the antibody and its complex when using an RDES cell lysate as a sample. Thus, similar to A549, RDES cells are capable of expressing MRP1.

3.2. Determination of relative amounts of MRP1 in RDES and A549 cell lysates

The developed CEIA method was used to determine the relative amounts of MRP1 in A549 and RDES cancer cells. As shown in Table 2, the relative amounts of MRP1 in the cells were measured after incubating the cells in DOX-free, DOX, and DOX/probenecid culture media for either 12 or 24 h. Probenecid, which is known to inhibit MRP1 [42], was employed since it has been reported to enhance the accumulation of anthracyclines in A549 and RDES cells [43]. The results show that both cell lines expressed MRP1, even in the absence of DOX, and that A549 contained more MRP1 than

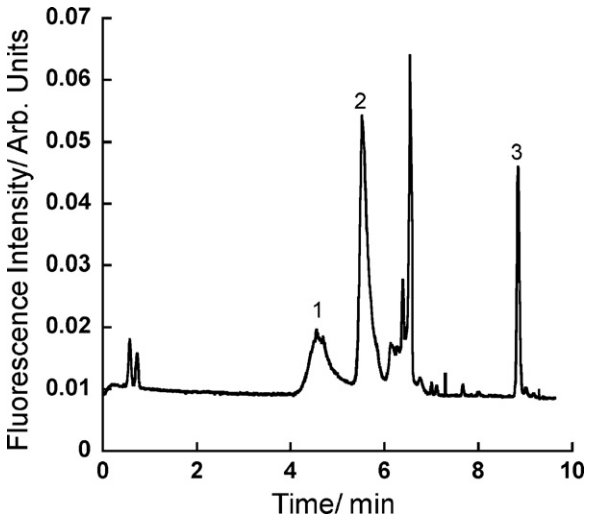

Fig. 3. The separation of anti-MRP1 and its immune complex. 1, anti-MRP1; 2, immune complex; 3, fluorescein. Sample: RDES cell lysate treated with DOX for 12 h, reaction time 60 min. Other conditions are the same as in Fig. 1.

RDES. Lung tissues express several ABC proteins in order to prevent the accumulation of harmful xenobiotics from inhaled air [44]. MRP1, which is known to cause MDR in many lung tumors [4], is localized in the basolateral surface, where it protects the lung tissues against airborne xenobiotics. Thus, even in the absence of DOX, A549 cells are expected to show relatively higher levels of MRP1 expression than RDES.

After treatment of cells with DOX for 12 h, the expression of MRP1 increased in both cell types, but to different extents: RDES showed a greater increase (57%) than A549 (29%), although the total amount was less than A549. The levels of expression of MRP1 did not differ between exposure of 12 and 24 h to DOX in either A549 or RDES. A nearly constant expression of MRP1 between the 12 h and 24 h incubation accompanied by decrease in DOX accumulation suggest that drug efflux can still occur provided that MRP1 has attained a certain level of expression. Generally, these results are in agreement with previous works [45,46], in which anthracyclines, including DOX and epirubicin, were reported to induce MRP1 expression in lung cancer cells. The MRP1 expression of the cells treated with DOX was similar to that of the cells treated with DOX/probenecid for 24 h incubation. It is interesting to note that the cells treated with DOX/probenecid showed a higher expression of MRP1 in 12 h than 24 h-incubation for both A549 and RDES. This implies that MRP1 expression is also

Table 2
The levels of MRP1 expression and the amount of accumulated DOX in cancer cells.

Cell type	Treatment	Relative amount of MRP1/protein content (nmoles/mg)	Amount of DOX/protein content (μmoles/mg)
A549	F	76.4 ± 2.4	0
	A-12	98.8 ± 4.0	0.42
	A-24	94.0 ± 3.2	0.26
	AI-12	144 ± 5.8	0.99
	AI-24	90.0 ± 3.2	–[a]
RDES	F	43.2 ± 0.2	0
	A-12	68.0 ± 3.0	1.15
	A-24	70.0 ± 0.2	0.99
	AI-12	98.0 ± 4.4	1.56
	AI-24	73.6 ± 2.6	–[a]

F, DOX free; A-12, 12 h incubation with DOX; A-24, 24 h incubation with DOX; AI-12, 12 h incubation with DOX and probenecid; AI-24, 24 h incubation with DOX and probenecid.

[a] Amounts of DOX were not determined for AI-24.

affected by inhibitors, although the reason for the observed down-regulation after 24 h treatment with DOX/probenecid is unknown. Similar down-regulation of P-glycoprotein was observed in rat astrocytes with protracted treatment at a high concentration of DOX (500 ng mL^{-1}, 48 h) [47]. Therefore, a high concentration of a substrate for an ABC protein may induce up-regulation and subsequent down-regulation, although further investigation is necessary to clarify the mechanism involved.

To further evaluate the method, the relative amounts of MRP1 were compared with intracellular DOX concentration, in which the amount of DOX was determined using the same CE-LIF system and employing a previously developed method [34]. Several studies have shown that the expression of MRP1 lowers the sensitivity of the cells towards DOX [20,48]. The lowered sensitivity to DOX would be induced by efflux of DOX through over-expressed MRP1. Therefore, the results of the present study are consistent with the aforementioned findings [20,48] since increase of MPR1 expression and reduction of DOX concentration were observed simultaneously when either A549 or RDES was treated with DOX upon incubation for 12 or 24 h (Table 2). However, the amounts of DOX that accumulated in the presence of probenecid, in both A549 and RDES, did not reflect an increase in MRP1 expression. A similar observation was made by Rajagopal et al. [49] when they examined MRP1 activity using transient expression of fluorescently tagged MRP1. This observation may be ascribed to probenecid being an MRP1 substrate, which is therefore effluxed at the expense of DOX. Thus, an increase in MRP1 causes a higher efflux in probenecid than in DOX, leading to a modest increase in the intracellular DOX concentration.

4. Concluding remarks

A CEIA-LIF method for relative quantification of MRP1 was developed. The method is useful as a quick analytical tool for relative quantification of MRP1 by virtue of its simplicity and shorter analysis time. The method's reliability has been demonstrated by the similarity of its results to those obtained by other established methods. The present study also demonstrates that CEIA-LIF can be used to separate higher-mass proteins (>170 kDa), and, hence, can be used to investigate ABC and other superfamilies of proteins, which play crucial roles in cell activities. Because of the method's ability to measure the kinetics of complex formation, more comprehensive investigations of the rate of complexation can be designed to gain further understanding of how to control the functioning of transporter proteins.

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